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THE RELATION OF WORK AND OXYGEN CONSUMPTION IN ISOLATED STRIPS OF CAT AND RAT MYOCARDIUM

By W. J. WHALEN

From the Department of Physiology, College of Medicine, State University of Iowa, Iowa City, Iowa, U.S.A.

(*Received* 4 *July* 1960)

In previous studies (Whalen, 1959, 1960) it was reported that initial length and, to a much greater extent, frequency of the isometric contractions were the factors which most significantly affected the oxygen consumption of excised strips of parallel-fibred mammalian myocardium. The amount of the developed or resting tension did not appear to be consistently related to the Q_{O_2}. Recently a sensitive isotonic gauge was devised in order that the influence of shortening and load on the Q_{O_2} could be assessed. If the energetics of mammalian heart muscle and amphibian skeletal muscle are similar, additional oxygen should be consumed (see Fenn, 1923; Fischer, 1931; Hill, 1953). The experimental results presented here are also compared with the results from studies on the effect of work and tension on the oxygen consumption of the intact heart.

METHODS

One of the trabeculae carneae or a papillary muscle of the cat (Cattell & Gold, 1938) or rat (Ullrich & Whitehorn, 1956) was excised and fastened to the muscle holder as previously described (Whalen, 1957). During dissection the muscle was constantly aerated with 98% oxygen and 2% (v/v) carbon dioxide.

The respiration chambers previously described (Whalen, 1957) permit the simultaneous measurement of muscle tension and oxygen consumption. Modifications of the chambers have improved the accuracy of the measurement of the oxygen uptake (see Fig. 1). A compensating chamber has been added and oxygen consumption is now measured volumetrically, essentially as described by Scholander & Edwards (1942) and modified by Wennesland (1949). The mercury seal, through which a monofilament nylon thread transmits muscle tension to a Statham strain-gauge (or isotonic lever), was also modified by embedding a ring of nickel around the mercury well. Previously the essentially non-wettable plastic surface permitted the occurrence of a small unpredictable leak around the mercury 'seal'. The nickel ring, which is wetted by the mercury, has eliminated this source of error.

The muscle and weights were attached to the isotonic lever as shown in Fig. 1. To reduce the effect of inertia the weight was hung 1 cm from the potentiometer shaft and the muscle attached 2 cm distant. (In the text reference will be made to the *actual* load on the muscle, one half of the attached weight.) In considering whether the contraction was indeed isotonic, the reaction force on the muscle was calculated on the basis of an estimated maximum acceleration of 150 cm/sec^2 seen at the lightest muscle load of 0·5 g. The moment of inertia (I)

of the lever was obtained from the formula for a thin homogeneous rod, $I = \frac{1}{12} \times 0{\cdot}32\,g \times (4)^2 = 0{\cdot}43\,g.cm^2$; the total moment ($I$) being $1{\cdot}43\,g.cm^2$. With an angular acceleration (α) of 75 radians/sec^2 the force due to acceleration was about 11 % of the force due to gravity. (With heavier loads the reaction force would be somewhat less.) When these calculations were checked empirically by attaching the lower end of the muscle to an isometric gauge, the departure from isotonicity was found to be about 15 %.

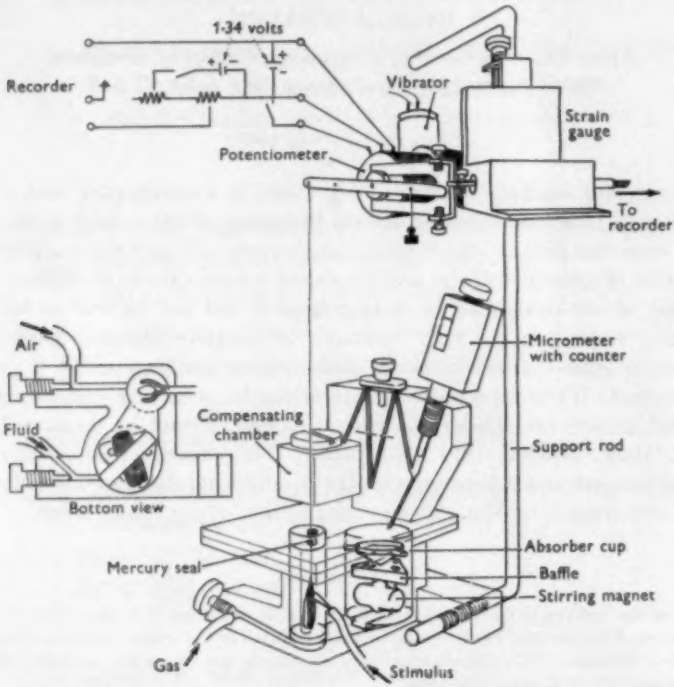

Fig. 1. Drawing of chamber and isotonic gauge. The potentiometer is a Giannini Microtorque, model no. 85113. When vibrated at 400/sec the starting torque is nearly zero. With the Offner Dynograph recorder used, movements of the tip of the lever arm could be measured with an accuracy of 0·025 mm.

The work done was calculated from the final height to which the load was raised, since, except for any frictional force, the kinetic energy imparted to the system would be converted to potential energy during deceleration. Since the acceleration did not exceed that due to gravity, no 'over-shoot' would be expected, and changes in muscle length could also be measured from the total excursion of the recording pen.

Three chambers filled with 8 ml. of Feigen's solution (Feigen, Masuoka, Thienes, Saunders & Sutherland, 1952) were placed in a constant-temperature bath held at $31{\cdot}7 \pm 0{\cdot}01°$ C. Two chambers contained tissue, the other served as a thermobarometer and as an additional control for the oxygen uptake by the diethanolamine CO_2 'absorber' (Pardee, 1949; Krebs, 1951). After gassing the chambers with 98 % O_2 and 2 % CO_2 (v/v) the gas was turned off and the chambers were allowed to equilibrate for 45 min.

In a preliminary series of experiments (series *A*) the muscles were free-loaded during the periods of isotonic contraction. A 1 g load was placed on each muscle (see Fig. 1) and the two muscles allowed to extend passively for 10 min, at which time one muscle was then connected to the strain-gauge for registration of isometric tension. Stimulation of both muscles with biphasic, supramaximal pulses of 1 msec duration at 30/min was then begun through the main platinum electrodes lying alongside each muscle. In successive periods of 50 min each, during which oxygen consumption and contractile force, or shortening, were recorded the type of contraction was alternated. Ten-minute 'change-over' intervals were interspersed between the experimental periods to allow for any oxygen debt or lag in the system (Whalen, 1957). A day's run usually consisted of two or three isometric periods and two or three isotonic periods.

In the second series of experiments (*B*) the muscles were after-loaded. Thus, the initial length of the muscle was held constant during the paired periods of isotonic and isometric contraction. In addition, a wide range of loads was used over a wide range of initial lengths, and the experimental periods were shortened to 30 min. Also, the strain-gauge was not used in series *B* since it did permit a small amount of shortening. Instead, the isotonic lever was held rigidly by a set-screw, which limited the amount of shortening to less than 3%. At the end of the experiments the muscles were measured, blotted, and weighed immediately. The tissues were dried overnight at 100° C and weighed again. The dry weight was $23{\cdot}0 \pm 4\%$ (S.D.) of the wet weight. Q_{O_2} was calculated in the usual manner, it being expressed as $\mu l.O_2$ (s.t.p.)/mg dry wt./hr.

In series *A* (and *C*) only muscles which measured less than 1·2 mm in diameter were employed. In series *B* the measured diameter of the 32 muscles used ranged from 0·4 to 1·8 mm, with a mean of approximately 1·1 mm.

The critical diameter for adequate oxygen diffusion. Calculation of the critical radius for oxygen diffusion was carried out by Hill's (1928) equation, $r' = \sqrt{(4Ky_e)/a}$ where K is the diffusion coefficient of oxygen through the tissue, y_e is the external oxygen tension, and a is the oxygen consumption in ml. O_2/ml. wet tissue/min. The Krogh (1918) diffusion coefficient as used by Hill (1928), extrapolated to 31·7° C, gave a value of $1{\cdot}57 \times 10^{-4}$ (cf. Creese, Scholes & Whalen, 1958). To determine the appropriate value for the oxygen consumption, a curve was drawn relating oxygen uptake and the cross-sectional area as calculated from the net weight and length. In this calculation, which gave a somewhat higher value for the diameter, the specific gravity was assumed to be 1·05 as found by Creese (1954) for the rat diaphragm. Up to a cross-sectional area of 1·35 mm^2 the trend of the oxygen consumption was upward, though the rise was not significant. With thicker muscles the Q_{O_2} fell somewhat. It was also observed that most of the thinner muscles showed less decrement in contractile force with time. Thus, the oxygen consumption of 0·023 ml./ml. wet tissue/min obtained from the twelve thinnest muscles in series *B* was chosen as being the most accurate value. The value for y_e was taken as 0·98. Substituting these values in the Hill equation revealed that the diameter of the muscle should not have exceeded 1·0 mm in order to insure adequate diffusion through the entire muscle. It is obvious that some of the muscles in series *B* must have become anoxic at the core, but the results from thick and thin muscles were qualitatively similar.

RESULTS

Table 1 summarizes the data from the first series of twelve experiments with the cat papillary muscle. The mean amount of shortening during isotonic contraction in this series was 1·4 mm, a change of about 12·2% from the mean final measured resting length of 11·6 mm. Since the muscles were free-loaded during the isotonic periods, some extension over the original unstretched length occurred during the course of the experiments.

The extension amounted to about 30% of the unstretched length at the beginning. Most of the extension occurred in the first 10 min after adding the weight. This degree of stretch is below the point at which efficiency is maximum (see below), but about at the point where contraction amplitude is maximum, the 'optimum length' (Whalen, 1960).

Table 1 shows that regardless of the statistical method of treatment the Q_{O_2} during periods of isotonic contraction was significantly lower than during periods of isometric contraction. It would appear that any extra energy liberated as a result of shortening or work does not increase the total oxidative metabolism of heart muscle.

TABLE 1. Comparison of the Q_{O_2} during isotonic and semi-isometric contraction at frequency (F) of 30/min. Paired data from each of twelve strips were averaged in determination of percentage change. The absolute values are unpaired, and here N refers to the number of determinations

		Q_{O_2} (%)		
N	F	Isotonic (1 g wt)	Isometric	P
12	30	100	116 ±3·0 (S.E.)	> 0·01
		Q_{O_2} (absolute values)		
30	30	3·23 ± 0·15	3·73 ± 0·18	0·05

In order to determine whether this lack of correspondence between work or shortening and oxygen consumption was limited to cat myocardium, rat trabeculae carneae were tested. The experimental conditions were the same as in series *A*, except that the bath temperature was 28° C and the stimulation rate was 60/min. The mean Q_{O_2} of twelve muscles for the isotonic periods was 6·0 ± 0·48 (S.E.) and 5·9 ± 0·40 for the isometric periods—obviously not significantly different.

The question arose as to whether the apparent lack of direct correspondence between oxygen consumption and work or shortening was obscured at the near-optimum length used. By extending the range of length and loads to include both sides of the optimum length an increased 'isotonic' Q_{O_2} might be seen. Fenn (1923) did find that the extra heat passed through a maximum at about the point of maximum work output, after which both work and heat declined to the isometric level (see also Fenn & Latchford, 1933).

The results from series *B* are shown in Fig. 2. Since cat papillary muscles and trabeculae carneae responded similarly, the data were pooled. In none of the sixteen experimental conditions was the isotonic Q_{O_2} significantly different, at the 5% level of confidence, from the isometric level. This was true even when analysed on the basis of percentage change to eliminate inter-individual variability. The points for the lowest load at each length are replotted in Fig. 3, to reveal more clearly the increase in Q_{O_2} accom-

panying an increase in both the length and the load. That this increase is mainly due to the increased length is illustrated in Fig. 4, in which the results obtained at the 1250 mg point are plotted to reveal the effect of increasing length at a constant load. Here the isotonic and isometric Q_{O_2}

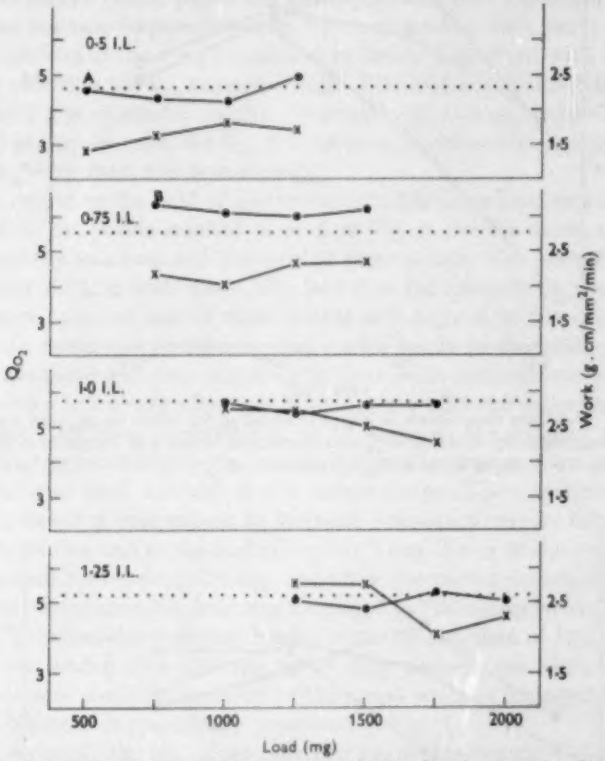

Fig. 2. Data from 22 cat papillary muscles and 10 trabeculae carneae stimulated at 30/min. Each point plotted represents 11–15 determinations. The S.E. of the Q_{O_2} value of each point ranged from 0·37 to 0·67. Work, ×, is expressed as g.cm/mm²/min. ●—●, isotonic Q_{O_2}; ------, isometric Q_{O_2}. L.L. refers to the initial length attained with the various loads (see Methods).

rise together. The increase in Q_{O_2} with length is about that to be expected from previous work (Whalen, 1960). The declining limb with extensive stretch was not, however, seen in the earlier work on heart muscle (Whalen, 1960), although it was found in rat diaphragm muscle (Whalen, Dernberg & Jenden, 1958). Hill (1958) found a decline in the heat production beyond resting length during isometric twitches of the frog's sartorius muscle. The decline in Q_{O_2} with greater extensions in the present experi-

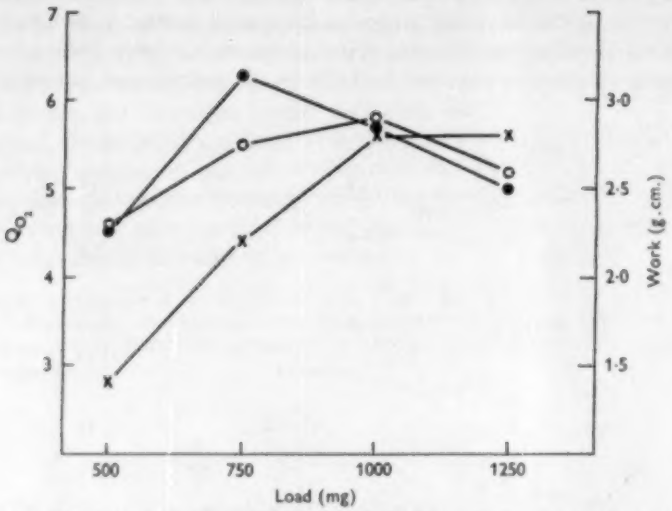

Fig. 3. Plot from data shown in Fig. 2 illustrating the effect on Q_{O_2} and work of increasing length up to about 60 % over the relaxed length and weight from 500 to 1250 mg. × work; ● isotonic Q_{O_2}; ○ isometric Q_{O_2}.

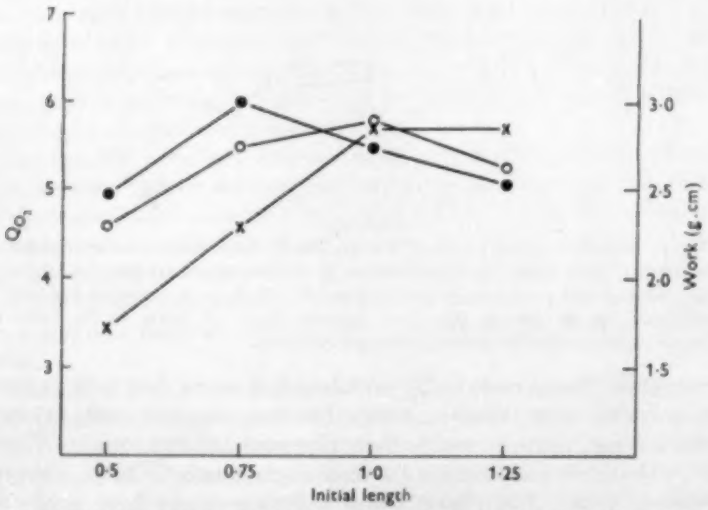

Fig. 4. Plot from data in Fig. 2 showing the effect on Q_{O_2} and work of increasing length at constant load of 1250 mg; symbols as in Fig. 3.

ments is almost certainly due to a fault in the experimental design, in that the largest extensions were almost invariably used in the last periods of the day's run when the Q_{O_2} had fallen about 20%. For the present it is most important to know that the work performance had exceeded maximum where in Fenn's (1923) paper the greatest excess heat was reported. That such was the case is apparent from Fig. 2, where the work curve is seen to reach a plateau at the 1·0 g length and to decline somewhat with increasing weight, although the Q_{O_2} remained high. Five additional experiments with a load of 3 g gave similar results. Summarizing, it is apparent that with a twofold change in work the Q_{O_2} did not vary significantly; nor did the isotonic Q_{O_2} vary from the isometric Q_{O_2}.

With regard to the heat of shortening it might have been expected to be greatest at the points marked *A* or *B* in Fig. 2, for the mean shortening (1·3 mm) was maximal and identical at these points. The change in length was about 13% in both cases. The fact that the isotonic Q_{O_2} was close to the lowest value at one of these points and highest in the other argues against an extra energy release attributable solely to shortening.

Because there was some disparity in the results between series *A* and *B*, and in order to test the effects of fatigue, six additional experiments were performed. In these experiments, as in series *A*, the muscles were free-loaded during isotonic periods, but the range of loads was extended and the set-screw was used instead of the strain-gauge. The experiments were designed to allow comparison to be made between a certain initial length at the beginning and at the end of the day's run. Three of the cat papillary muscles were loaded with 500 mg., and after two paired periods of isotonic–isometric contraction the load was increased to 750 mg for two more paired periods. Two muscles were first loaded with 750 mg, then at 1250 mg. One muscle was loaded with 1250 mg for all eight periods, and since the decrement was zero could be included in the graph without unduly biasing the results. Series *C* is graphically presented in Fig. 5.

As in series *A*, the Q_{O_2} of isotonic periods is significantly below that of the isometric periods. It apparently does not matter whether the muscle is 'fresh' or 'used'. Nor is it only at the 1·0 g length that free-loaded muscles contracting isotonically consumed less oxygen than during isometric contraction at the same length.

In series *C* the Q_{O_2} did not decrease with time under conditions in which some extension of the muscle took place. This fact suggests that the decrement in Q_{O_2} with time, which occurs at constant length, is almost exactly balanced in free-loaded muscles by the increment in Q_{O_2} with increasing initial length.

As in the afterloaded series (*B*), the work increased with increasing load, although the plateau of maximum work appears to have been reached at

about the 0·75 g point rather than at the 1·0 g point as before. Work and Q_{O_2} at the lower weight range showed a negative relationship, even though a greater Q_{O_2} might have been expected on the basis of greater initial length alone (Whalen, 1960).

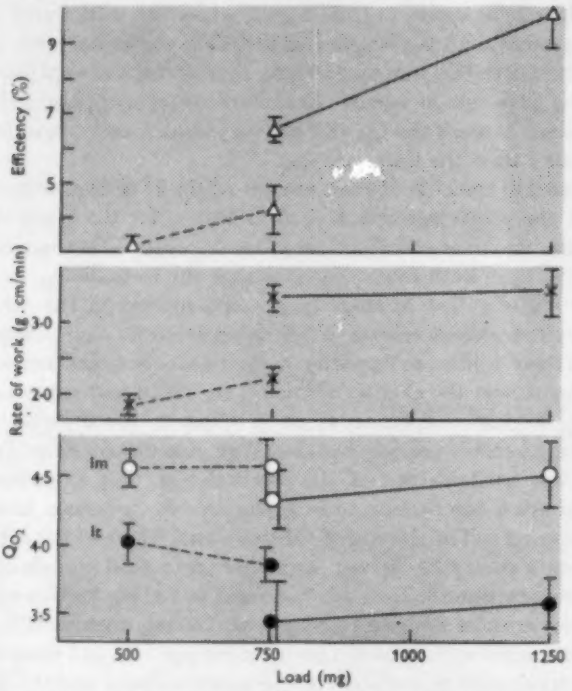

Fig. 5. Mean Q_{O_2}, total work and efficiency of six cat papillary muscles; three with light loads (------), and three different muscles with heavier loads (——). The S.E. of each point is represented by the vertical bars. Symbols as in Fig. 3. (For further explanation, see text.)

Percentage efficiency

The over-all percentage efficiency was calculated according to the equation

$$\% E = 100 \times \frac{\text{g.cm (total } W\text{/min).}}{\text{g.cm (total } O_2\text{/min)}}$$

It was assumed that the caloric equivalent of O_2 was 4·85 kcal/l. (Lorber, 1953). Conversion of the heat units to units of work gives a value of 213 g.cm/μl. O_2. The mean over-all efficiency at which work was done in series *A* was 6·9 %. In series *B* the efficiency ranged from a mean of 3·9 %

at the lightest load and shortest initial length to 6·3 % at the 1·25 g initial length loaded with 1·5 g. With larger loads at the latter initial length efficiency declined to 5·2 %. The lower efficiencies in series *B* were partly due to the influence of the thicker muscles. The mean efficiency of the muscles less than 1·35 mm² in cross-section was 9·7 %; about the same as in series *C* (see Fig. 5). These calculations of the over-all efficiency include the fraction of the metabolism necessary for maintenance at rest. After subtracting the resting oxygen consumption, obtained by extrapolating from data of previous experiments (Whalen 1960), the calculated external efficiencies are from two to four times higher. The thin strips in series *B* averaged 17·9 %. In series *A* and *C* the mean external efficiencies were 41 and 33 % respectively.

DISCUSSION

It would appear from the present results that there may be major differences between the energetics of isolated frog skeletal muscle and isolated mammalian cardiac muscle. These differences are most evident when comparison is made with heat measurements during tetanic contractions of frog sartorius muscle (e.g. Fenn, 1923; Abbott, 1951). The most closely related studies, however, are those of Fischer (1931), who measured the oxygen consumption of frog sartorius during a series of single twitches. The differences from my results are not so striking. Fischer reported that while he found a 13 % increase in oxygen consumption with work, it only occurred at small shortenings with relatively heavy loads. As in the current studies, with large shortenings much less O_2 was consumed. Fischer who, of course, had not the later experiments of Hill (1949*a*, *b*; 1953) with which to draw comparison, concluded that, 'with single twitches the amount of shortening occurring during the contraction is of much greater influence than the work done'. He called upon the experiments of Hill (1925; see Hill, 1958) showing that the heat liberated during tetanic contraction increased as muscle length increased, as has recently been shown to hold true for twitches of isolated heart muscle also (Whalen, 1960). Thus, since the mean muscle length was shorter during isotonic contraction the energy liberated would be expected to be less. Quantitatively this explanation appears to fall short of accounting for the significantly lower 'isotonic' Q_{O_2} in series *A* and *B*, although it is still an open question.

The possibility was entertained that the extra oxygen liberated with work was too little to be measured by the present method. The amount of extra oxygen, above the isometric, which might have been expected during the isotonic periods was calculated from the data of Fenn (1923). It was assumed that the temperature difference (approximately 7° compared with 32° C) is negligible. Fenn (1923) found that efficiency did not vary

within a range of 0–15° C. However, efficiency of the dog heart increased about 50 % with a change from 37° to 27° C (Reissman & Van Citters, 1956).

In series *A* the muscles lifted 1 g an average distance of 1·4 mm at a rate of 30 times/min for 50 min or a total of 1500 times. Thus,

$$\frac{\text{total work (g.cm)}}{\text{O}_2 \text{ equiv (213 g.cm/mm}^3\text{)}} = 0{\cdot}98 \text{ mm}^3 \text{ O}_2,$$

and since additional heat above the work equivalent was given off in the experiments of Fenn (1923), to the extent of 80 %, the initial O_2 equivalent would be expected to be about 1·75 mm^3. The total extra O_2 would be at least twice this amount (Hill, 1939*a*) to allow for recovery, or 3·5 mm^3.

Since the standard deviation of a series of 50 min determinations on one strip was no more than 0·60 mm^3 this difference should have been detected.

The heat of shortening is a quantity about which there is less controversy. Hill (1949*b*) found that, provided shortening remained constant, the load on a frog sartorius muscle during a twitch could be varied tenfold without affecting the amount of heat liberated. In a later paper (1953) he did find with an inertia lever that a fivefold increase in work increased the heat liberated by about 20 % above the shortening heat.

A number of papers (e.g. Hill, 1949*a*) have established that in amphibian skeletal muscle the heat of shortening per centimetre is approximately 400 g.cm/cm^2 cross-section. Abbott (1951) and Abbott & Wilkie (1953) extended these observations to show that this constant was applicable through a wide range of shortening. Calculation of the extra oxygen equivalent to the heat of shortening showed that an increment of about 10 mm^3 O_2 might have been expected in series *A*. If external shortening had been as much as 3 %, the excess O_2 due to shortening would have been about 7·5 mm^3 O_2—still an easily measurable difference.

It is obvious that the amount of internal shortening complicates the determination of shortening or work energy. On the basis of the increased extensibility of heart tissue it may be that internal shortening is greater than in frog skeletal muscle, in which internal shortening, according to Hill (1949*a*), can be as much as 5–10 % of the length. The data in the present paper provide no evidence on this point, however.

It is possible that the positive effect on Q_{O_2} of work or shortening is a characteristic peculiar to amphibian skeletal muscle. There is some information which indicates that the Q_{O_2} of the isolated rat diaphragm is not dependent on the work done (R. E. Smith & W. J. Whalen, unpublished). Also a recent paper by Fales, Heisey & Zierler (1960) showed that the number of stimuli rather than work or shortening best correlated with the oxygen consumption of the *in situ* gastrocnemius-plantaris muscle group

in the dog. However, Lee (1960) from his work on the relationship between isometric contraction and Q_{O_2} of the cat papillary muscle reached the conclusion that there were no basic differences from frog skeletal muscle.

The present results also give some indication that heart muscle may perform external work more efficiently than skeletal muscle. Fischer (1931) found a mean efficiency of 9 % for the frog sartorius muscle, after he had subtracted the resting O_2 uptake. On the basis of heat measurements Hill (1939*b*) found the efficiency of the initial process, exclusive of recovery, to be around 40 %; or 20 % for the entire cycle. He noted that the efficiency of human skeletal muscle appeared to be of the same order of magnitude. On the other hand, Bing & Michal (1959), after subtracting estimates of the resting O_2 consumption, calculated the efficiency of human and dog heart *in vivo* to be almost 40 %. The latter figure agrees with the estimates of external efficiency in series *A* and *C* found in the present study. It should, however, be mentioned that Lorber (1953), found relatively low values for the over-all efficiency in the isolated blood-perfused cat heart performing light work. Also, the uncertainty regarding the rate of the resting O_2 consumption makes any comparison hazardous. Furthermore, there is no evidence which shows that the resting O_2 consumption remains the same during activity as during rest.

In a previous paper (Whalen, 1960) some evidence was presented which suggested that, for isolated heart muscle (and isolated rat diaphragm (Whalen, Dernbergh *et al.* 1958)), it was the initial length rather than the developed or resting tension which determined the oxygen uptake per contraction, although total tension could not be eliminated as a contributing factor. Lee (1960), on the other hand, reported that resting tension, in the cat papillary muscle was a more significant factor than length. Our combined results lead to the tentative conclusion that, in isolated myocardium, a 'package' of energy is liberated with each beat which is directly proportional to the initial length, and apparently independent of work or shortening. The *in vitro* results further suggest that, in the intact animal, the heart rate should be the major determinant of the oxygen consumption (Whalen, 1960). These hypotheses in some respects are consistent with the results from recent *in vivo* experiments.

Recent research on the whole heart has tended to reduce emphasis on the role of fibre length or end-diastolic volume in the determination of the total energy exchange (see Starling & Visscher, 1927). Katz, Katz & Williams (1955) found in the relatively intact dog that large variations in left atrial pressure (and presumably end-diastolic volume) were not accompanied by corresponding variations in O_2 consumption. Sarnoff, Braunwald, Welch, Case, Stainsby & Macruz (1958) agree that factors other than

length predominantly influenced the Q_{O_2} in their isolated-supported heart preparation. According to Rushmer & Smith (1959) the maximum increase in heart size in their experiments on unanaesthetized dogs was 15%. If my findings (Whalen, 1960) can be accepted and applied to the intact organism, the increment in O_2 uptake which might be expected from this amount of distension would be only about 15%.

On the other hand, an increase in frequency over the normal range from 60 to 120 beats/min would double the O_2 consumption (Whalen, 1960). The graphs of Sarnoff *et al.* (1958) show that increasing the frequency alone called forth a substantial increase in the Q_{O_2}. Hoffmeister, Kreuzer & Schoeppe (1959) obtained in the dog heart when empty almost the same increase in O_2 consumption with frequency as that found by Whalen (1960).

Until quite recently there was general agreement that work was a major determinant of the O_2 consumption in heart muscle (cf. Evans & Matsuoka, 1914). However, Sarnoff *et al.* (1958), using their refined dog heart preparation, reported that myocardial Q_{O_2} bore little relation to the heart's external work *per se*. Salisbury, Bor, Lewin & Rieben (1959), using a heart–lung machine bypass, also found little or no change in oxygen consumption with large variations in cardiac work. Katz & Feinberg (1958), from results on a relatively intact mammalian preparation, reached a similar conclusion.

Although the selective manner in which the results from the intact heart were presented above may give the impression of complete accord with the *in vitro* results, this is not quite the case. Sarnoff *et al.* (1958) noted that, if frequency were held constant, large increases in O_2 consumption still occurred in direct proportion to increases in arterial pressure. Further experimentation led them to suggest that the area under the systolic pressure curve, 'the tension–time index, or T.T.I.', was the primary determinant of the 'Q_{O_2}' per beat. Thus, (heart rate) × (T.T.I.) most accurately predicted the total O_2 consumption. Katz & Feinberg (1958), stimulated by Sarnoff's studies, reanalysed their data from the intact dog and found that (heart rate) × (aortic pressure) yielded a very high correlation with myocardial O_2 consumption.

The major significance of aortic pressure or tension development is, therefore, at variance with the *in vitro* results. A possible explanation for the discrepancy is the following. In the studies of Sarnoff *et al.* (1958) a very high correlation between O_2 consumption and coronary blood flow was observed, as others had previously reported (e.g. Alella, Williams, Bolene-Williams & Katz, 1955), although occasional deviations from this relationship have been found in the dog heart subjected *in situ* to stress (Katz *et al.* 1955). In an analysis of the determinants of coronary flow Braunwald, Sarnoff, Case, Stainsby & Welch (1958) came to the conclusion that aortic

pressure was the dominant factor. Thus, both aortic pressure and myocardial O_2 consumption are significantly correlated with the coronary blood flow. This relationship might be interpreted in at least two ways or a combination of both: (1) greater aortic pressure means greater tension development and thus more oxygen consumed, coronary flow being adjusted to the demand for oxygen, or (2) coronary flow primarily determines the oxygen consumption, tension being relatively unimportant. The latter view would tend to eliminate the disparity between the results from the *in vitro* and intact heart experiments.

There is some evidence which supports the hypothesis that coronary flow is a major determinant of myocardial oxygen consumption. For example, Gregg, Rayford, Khouri, Kattus & McKeever (1957) demonstrated in dogs that by increasing the coronary perfusion pressure by 5–35 mm Hg myocardial oxygen uptake increased 20–70 %, although there was no change in heart rate, cardiac output or aortic pressure. When the coronary perfusion rate was reduced below normal, the oxygen consumption was depressed with apparently little change in other functions. On the other hand, Berne (1958) did not always observe an increase in myocardial oxygen consumption with an increase in coronary blood flow in the fibrillating dog heart. Wegria, Nakano, McGiff, Muraviev, Zekert & Blumenthal (1959) increased coronary flow with ERL-239, which produced no change, or lowering, of arterial pressure, cardiac output, and work; yet they found a significant increase in myocardial O_2 consumption.

This hypothesis is tantamount to saying that the heart is in a continual state of oxygen debt, which it may be, depending on the definition of the term. Verzar (1912) may have been the first to suggest this hypothesis for skeletal muscle. The evidence is fragmentary but suggestive. In this sense the heart cannot incur an oxygen debt simply because heart muscle contains the essential ingredients to turn over oxygen at a much faster rate than it is normally supplied, turning the energy into heat unless an alternative pathway is available (or necessary). Should this assumption prove to be correct, it would mean that heart muscle differs from skeletal muscle, since O_2 consumption in the latter is independent of the O_2 tension down to extremely low pressures (Hill, 1948).

The question arises whether, in the present experiments, the oxygen supply was equal to the demand. This has been discussed under Methods (see also Whalen, 1957, 1960) and, except for the very thick muscles, answered affirmatively. If so, it becomes necessary to explain why the calculated values for Q_{O_2} are below most of the values for the *in vivo* and blood-perfused heart studies mentioned earlier. It is probable that the catechol amines present in the blood would contribute significantly to the metabolic demand (e.g. Whalen, 1957). Further, lactate and pyruvate

available in the blood-perfused heart might be expected to increase the oxygen consumption (Bernheim & Bernheim, 1944).

A complicating factor in the analysis of the over-all results is the possibility that the amount of the 'available' intracardiac catechol amines probably varies with the activity of the heart muscle (Whalen, Fishman & Erickson, 1958; Sarnoff, Mitchell, Gilmore & Remensnyder, 1960). If muscle tension (or stretch), no matter how produced, facilitates the 'spontaneous' intracardiac liberation, or use, of the catechol amines, the qualitative differences between our results and those from the intact heart preparation would tend to disappear. The attractive possibility of the more general applicability of this hypothesis must await further proof.

SUMMARY

1. By means of a newly developed microrespirometer the oxygen consumption and external work output of excised, parallel-fibred cat and rat trabeculae carneae and papillary muscles were simultaneously measured during isotonic contractions at a frequency of 30 to 60 per minute. In alternate periods shortening was limited to less than 3% of the initial length, and thus, essentially no external work was performed. A wide range of initial lengths and loads was used.

2. In experiments in which the muscles were free-loaded the Q_{O_2} during periods of isometric contraction was significantly higher than during periods of isotonic contraction. When the muscles were after-loaded there was no significant difference in the Q_{O_2} for the two types of contractions. No explanation was offered for the disparity of the results between free-loaded and after-loaded experiments.

3. Work and Q_{O_2} were found not to be correlated even at the point of maximum efficiency and beyond it; nor was the amount of shortening a significant factor in determining the Q_{O_2}. It was suggested that the lower Q_{O_2} often seen during isotonic periods may have been partly due to the shorter mean length during contraction.

4. The efficiency for doing external work was estimated, and found to be in the same range as that reported for the whole heart, and possibly higher than for skeletal muscle.

5. The results indicated that there may be major differences in the energetics of isolated mammalian heart muscle and isolated amphibian skeletal muscle. It was suggested that for isolated heart muscle, a 'package' of energy is liberated per beat which is directly proportional only to the initial length—or, possibly, the mean length—during contraction. In the intact organism frequency would be expected to be the major determinant of myocardial O_2 consumption.

6. The tentative hypotheses put forth in the preceding paragraph were

found to be consistent with recent results obtained from whole-heart preparations, with the exception that, in the whole heart, muscle tension has been found to be the major determinant of the 'Q_{O_2}' per beat. The suggestion was made that coronary flow may be one of the parameters which determine the O_2 consumption of the heart, and that variations in the coronary flow might be responsible for the lack of agreement regarding the effect of tension on the Q_{O_2}.

7. A possible relation between muscle activity and intracardiac catechol amine liberation was also discussed.

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REFERENCES

ABBOTT, B. C. (1951). The heat production associated with the maintenance of a prolonged contraction and the extra heat produced during large shortening. *J. Physiol.* **112**, 438–445.

ABBOTT, B. C. & WILKIE, D. R. (1953). The relation between velocity of shortening and the tension–length curve of skeletal muscle. *J. Physiol.* **120**, 214–223.

ALELLA, A., WILLIAMS, F. L., BOLENE-WILLIAMS, C. & KATZ, L. N. (1955). Inter-relation between cardiac oxygen consumption and coronary blood flow. *Amer. J. Physiol.* **183**, 570–582.

BERNE, R. M. (1958). Effect of epinephrine and norepinephrine on coronary circulation. *Circulation Res.* **6**, 644–655.

BERNHEIM, F. & BERNHEIM, M. L. C. (1944). The effects of various conditions on the respiration of rat heart muscle *in vitro*. *Amer. J. Physiol.* **142**, 195–199.

BING, R. J. & MICHAL, G. (1959). Myocardial efficiency. *Ann. N.Y. Acad. Sci.* **72**, 555–558.

BRAUNWALD, E., SARNOFF, S. J., CASE, R. B., STAINSBY, W. N. & WELCH, G. H., Jr. (1958). Hemodynamic determinants of coronary flow: effect of changes in aortic pressure and cardiac output on the relationship between myocardial oxygen consumption and coronary flow. *Amer. J. Physiol.* **192**, 157–163.

CATTEL, McK. & GOLD, H. (1938). The influence of digitalis glycosides on the force of contraction of mammalian cardiac muscle. *J. Pharmacol.* **62**, 116–125.

CREESE, R. (1954). Measurement of cation fluxes in rat diaphragm. *Proc. Roy. Soc.* B, **142**, 497–513.

CREESE, R., SCHOLES, N. W. & WHALEN, W. J. (1958). Resting potentials of diaphragm muscle after prolonged anoxia. *J. Physiol.* **140**, 301–317.

EVANS, C. L. & MATSUOKA, Y. (1914). The effect of various mechanical conditions on the gaseous metabolism and efficiency of the mammalian heart. *J. Physiol.* **49**, 378–405.

FALES, J. T., HEISEY, S. R. & ZIERLER, K. L. (1960). Dependency of oxygen consumption of skeletal muscle on a number of stimuli during work in the dog. *Amer. J. Physiol.* **198**, 1333–1342.

FEIGEN, G. A., MASUOKA, D. T., THIENES, C. H., SAUNDERS, P. R. & SUTHERLAND, G. B. (1952). Mechanical response of the isolated electrically driven rat ventricle strip. *Stanford med. Bull.* **10**, 27–31.

FENN, W. O. (1923). A quantitative comparison between the energy liberated and the work performed by the isolated sartorius muscle of the frog. *J. Physiol.* **58**, 175–203.

FENN, W. O. & LATCHFORD, W. B. (1933). The effect of muscle length on the energy for maintenance of tension. *J. Physiol.* **80**, 213–219.

FISCHER, E. (1931). The oxygen consumption of isolated muscles for isotonic and isometric twitches. *Amer. J. Physiol.* **96**, 78–88.

GREGG, D. E., RAYFORD, C. R., KHOURI, E. M., KATTUS, A. A. & MCKEEVER, W. P. (1957). Effect of alteration of coronary perfusion pressure on oxygen uptake of left myocardium. *Circulation*, **16**, 888.

HILL, A. V. (1925). Length of muscle, and the heat and tension developed in an isometric contraction. *J. Physiol.* **60**, 237–263.

HILL, A. V. (1928). The diffusion of oxygen and lactic acid through tissues. *Proc. Roy. Soc.* B, **104**, 39–96.

HILL, A. V. (1939*a*). Recovery heat in muscle. *Proc. Roy. Soc.* B, **127**, 297–307.

HILL, A. V. (1939*b*). The mechanical efficiency of frog's muscle. *Proc. Roy. Soc.* B, **127**, 434–451.

HILL, A. V. (1949*a*). The heat of activation and the heat of shortening in a muscle twitch. *Proc. Roy. Soc.* B, **136**, 195–211.

HILL, A. V. (1949*b*). Work and heat in a muscle twitch. *Proc. Roy. Soc.* B, **136**, 220–228.

HILL, A. V. (1953). A reinvestigation of two critical points in the energetics of muscular contraction. *Proc. Roy. Soc.* B, **141**, 503–510.

HILL, A. V. (1958). The relation between force developed and energy liberated in an isometric twitch. *Proc. Roy. Soc.* B, **149**, 58–62.

HILL, D. K. (1948). Oxygen tension and the respiration of resting frog's muscle. *J. Physiol.* **107**, 479–495.

HOFFMEISTER, H., KREUZER, H. & SCHOEPPE, W. (1959). Der Sauerstoffverbrauch des stillstehenden, des leerschlagenden und des flimmernden Herzens. *Pflüg. Arch. ges. Physiol.* **269**, 194–206.

KATZ, L. N. & FEINBERG, H. (1958). The relation of cardiac effort to myocardial oxygen consumption and coronary flow. *Circulation Res.* **6**, 656–669.

KATZ, L. N., KATZ, A. M. & WILLIAMS, F. L. (1955). Metabolic adjustments to alterations of cardiac work in hypoxemia. *Amer. J. Physiol.* **181**, 539–549.

KREBS, H. A. (1951). The use of 'CO_2 buffers' in manometric measurements of cell metabolism. *Biochem. J.* **48**, 349–359.

KROGH, A. (1918). The rate of diffusion of gases through animal tissues, with some remarks on the coefficient of invasion. *J. Physiol.* **52**, 391–408.

LEE, K. S. (1960). The relationship of the oxygen consumption to the contraction of the cat papillary muscle. *J. Physiol.* **151**, 186–201.

LORBER, V. (1953). Energy metabolism of the completely isolated mammalian heart in failure. *Circulation Res.* **1**, 298–311.

PARDEE, A. B. (1949). Measurement of oxygen uptake under controlled pressures of carbon dioxide. *J. biol. Chem.* **179**, 1085–1091.

REISSMAN, K. R. & VAN CITTERS, R. L. (1956). Oxygen consumption and mechanical efficiency of the hypothermic heart. *J. appl. Physiol.* **9**, 427–430.

RUSHMER, R. F. & SMITH, O. A., Jr. (1959). Cardiac control. *Physiol. Rev.* **39**, 41–68.

SALISBURY, P. F., BOR, N., LEWIN, R. J. & RIEBEN, P. A. (1959). Effects of partial and of total heart-lung bypass on the heart. *J. appl. Physiol.* **14**, 458–463.

SARNOFF, S. J., BRAUNWALD, E., WELCH, G. H., Jr., CASE, R. B., STAINSBY, W. N. & MACRUZ, R. (1958). Hemodynamic determinants of oxygen consumption of the heart with special reference to the tension-time index. *Amer. J. Physiol.* **192**, 148–156.

SARNOFF, S. J., MITCHELL, J. H., GILMORE, J. P. & REMENSNYDER, J. P. (1960). Homeometric autoregulation in the heart. *Circulation Res.* **8**, 1077–1091.

SCHOLANDER, P. F. & EDWARDS, G. A. (1942). Volumetric microrespirometer. *Rev. sci. Instrum.* **13**, 32–33.

STARLING, E. H. & VISSCHER, M. B. (1927). The regulation of the energy output of the heart. *J. Physiol.* **62**, 243–261.

ULLRICH, W. C. & WHITEHORN, W. V. (1956). A muscle column preparation from the rat's left ventricle. *Circulation Res.* **4**, 499–501.

VERZAR, F. (1912). The gaseous metabolism of striated muscle in warm-blooded animals. Part 1. *J. Physiol.* **44**, 243–258.

WEGRIA, R., NAKANO, J., MCGIFF, J. C., MURAVIEV, T., ZEKERT, H. & BLUMENTHAL, M. R. (1959). Effect of a new drug N-(2-endobicyclo-(2,2,1)-heptane) 1-diethylaminoacetamide hydrochloride (ERL-239), on mean arterial blood pressure, cardiac output, coronary blood flow, cardiac oxygen consumption, work and efficiency. *J. Pharmacol.* **126**, 164–167.

WENNESLAND, R. (1949). A volumetric apparatus for studies of tissue metabolism. In UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. *Manometric Techniques and Tissue Metabolism*, pp. 97–104. Minneapolis: Burgess Publishing Co.

WHALEN, W. J. (1957). Oxygen consumption and tension of isolated heart muscle during rest and activity using a new technique. *Circulation Res.* **5**, 556–561.

WHALEN, W. J. (1960). Some factors which influence the oxygen consumption of isolated heart muscle. *Amer. J. Physiol.* **198**, 1153–1156.

WHALEN, W. J., DERNBERG, E. & JENDEN, D. J. (1958). Interaction of stretch and ryanodine on oxygen uptake in the rat diaphragm. *Amer. J. Physiol.* **194**, 359–362.

WHALEN, W. J., FISHMAN, N. & ERICKSON, R. (1958). Nature of the potentiating substance in cardiac muscle. *Amer. J. Physiol.* **194**, 573–580.

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ACETYLCHOLINE AND THE SYMPATHETIC INNERVATION OF THE SPLEEN

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This paper is concerned with the role of acetylcholine-like substances in the sympathetic innervation of the spleen. The presence of cholinergic fibres in the sympathetic nerve supply to the cat's spleen was deduced by Burn & Rand (1960*c*) from observations on the response of the spleen of reserpine-treated cats to splenic nerve stimulation. Stimulation of the splenic nerve in a normal cat releases noradrenaline (Peart, 1949) and causes contraction of the spleen. But when the spleen is depleted of noradrenaline by reserpine (Burn & Rand, 1959*a*), splenic nerve stimulation produces either a smaller contraction than usual or a relaxation which is enhanced by eserine and abolished by atropine.

The innervation of the cat's spleen has been studied by Utterback (1944), who concluded that there was no parasympathetic innervation and that there were no ganglion cells in the spleen. Sympathetic denervation leads to a decrease in the noradrenaline content of the spleen (von Euler & Purkhold, 1951; Burn & Rand, 1959*a*), and von Euler (1956) concluded that the loss of noradrenaline on denervation could be explained if it were normally contained in the nerves. However, in another sympathetically innervated structure, the vessels of the rabbit's ear, denervation has been shown to cause a fall in acetylcholine content (Armin, Grant, Thompson & Tickner, 1953). It is well known that the spleen contains acetylcholine. This was first observed by Dale & Dudley (1929), although as yet no explanation has been given for its presence. In order to investigate the possibility that the acetylcholine might be associated with the splenic nerves, we have studied the effect of sympathetic denervation on the acetylcholine content of the cat's spleen.

Acetylcholine has been detected in the venous effluents after the stimulation of the sympathetic nerves to a number of structures: the sweat glands (Dale & Feldberg, 1934), the blood vessels of the dog's hind legs (Bülbring & Burn, 1935), and the vessels of the rabbit's ear (Burn & Rand,

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1960*c*). We have now shown that an acetylcholine-like substance appears in perfusates of cats' spleens after stimulation of the splenic nerves.

The drug hemicholinium causes failure of the response to cholinergic nerve stimulation (MacIntosh, Birks & Sastry, 1956; Reitzel & Long, 1959*a*, *b*; Wilson & Long, 1959) by inhibiting the synthesis of acetylcholine (MacIntosh *et al.* 1956; Gardiner, 1957). The effect of hemicholinium on responses to splenic nerve stimulation has been investigated. Observations have been made on the actions of acetylcholine in causing contractions of the spleen, to determine to what extent its effects mimicked those of splenic nerve stimulation.

METHODS

Determination of acetylcholine and noradrenaline contents of spleen. Cats were anaesthetized with ether and the spleens were removed. A segment of the spleen weighing 2 g was cut from its middle third. The portion was finely divided with scissors and ground with sand under 8 ml. of ice-cold 10% trichloroacetic acid. The mixture was transferred to a centrifuge tube, allowed to stand for 20 min, and then centrifuged at 4200 rev/min for 5 min. The supernatant was decanted and washed three or four times with 50 ml. ether. The remaining ether was removed by bubbling air through the extract. Acetylcholine was precipitated as the reineckate by the method of Shaw (1938) as modified by Bentley & Shaw (1952). The extract was placed in a 10 ml. centrifuge tube and 1 mg of choline chloride and 1·5 ml. of a freshly prepared saturated solution of ammonium reineckate in absolute alcohol were added for each 1 ml. of extract. The mixture was kept at $-10°$ C for 2 hr, and then centrifuged at 4200 rev/min for 2 min. The supernatant was discarded and the residue was dissolved in 16 ml. of 60% ethanol. Dilutions of the alcoholic extract (1:5 to 1:10) with distilled water were assayed against acetylcholine chloride on isolated segments of guinea-pig ileum suspended in a 10 ml. bath of Tyrode solution maintained at 32° C. No correction has been made for any losses of acetylcholine which may have occurred during the extraction procedure. The method was also used on ox spleen, which was found to contain 16 μg/g of acetylcholine, which was within the range of values of 4–30 μg/g found by Chang & Gaddum (1933).

The noradrenaline content of the spleen was determined in extracts prepared by the method of Burn & Rand (1959*a*), which were assayed on the blood pressure of the pithed rat, using a Statham strain-gauge manometer (Pennefather & Rand, 1960).

For experiments in which both acetylcholine and noradrenaline were determined, the central portion of the spleen was divided in a chequer-board fashion and alternating pieces were recombined for the separate extraction and estimation of each substance.

Sympathetic denervation of spleen. In cats under ether anaesthesia the abdomen was opened aseptically in the mid line and all the nerve fibres accompanying the coeliac axis were divided. Utterback (1944) has shown that the cat's spleen is completely denervated by dividing the coeliac plexus.

Isolated perfused spleen. Cats were anaesthetized with ether. The abdomen was opened and the main splenic artery, vein and nerve were dissected free. All other vessels running between the spleen and the greater curvature of the stomach, the omentum and the pancreas were doubly ligated and divided. The main splenic artery and vein were cannulated and the spleen was removed. A mixture of 95% O_2 and 5% CO_2 was bubbled through the perfusion fluid in the reservoir which was approximately 1 m above the spleen. The fluid was passed through coils immersed in a bath at 32° C before it reached the spleen. In some experiments in which the spleen was perfused with Tyrode solution the venous effluent was arranged to

superfuse a strip of guinea-pig ileum (Gaddum, 1953). In other experiments in which we wished to observe the effect of splenic nerve stimulation in contracting the spleen, we perfused with McEwen's (1956) solution and suspended the perfused spleen in a 50 ml. isolated organ bath so that its longitudinal contractions could be measured with a writing lever on a kymograph. In other experiments the venous outflow from the spleen was measured by counting drops with a Thorp (1948) impulse counter.

The splenic nerve was stimulated by one of two procedures: (*a*) a length of nerve was dissected and passed through a tube containing bipolar platinum electrodes, continually irrigated with the solution used to perfuse the spleen (Burn & Rand, 1960*a*); (*b*) a length of silver wire (0·008 in. (0·2 mm) diam.) was passed down the arterial cannula so that one end was near the tip of the cannula to form one electrode and a second electrode was attached to the tissue adjacent to the cannula. The nerve was thus stimulated by current traversing the splenic nerves which surround the artery. Square-wave stimuli of 2 msec duration from a constant voltage source at 5–50 V were used. Details of the frequency and the period of stimulation are given in Results.

Drugs were injected into the arterial cannula of the perfused spleen or into a 10 ml. bath containing a strip of spleen suspended in McEwen's solution at 32° C.

Cats were treated with reserpine by giving an intraperitoneal injection of reserpine 1·5–2·5 mg/kg dissolved in a 20% solution of ascorbic acid on each of the 2 days before the experiments.

Doses of noradrenaline are expressed as the base, acetylcholine as the chloride, bretylium as the tosylate (Darenthin), hemicholinium (α, α′ dimethylaminoethanol 4,4′ biacetophenone) as the dibromide and neostigmine as the methylsulphate.

RESULTS

Acetylcholine equivalent of normal and denervated cat spleen

The mean acetylcholine equivalent of spleens from 18 cats was 0·472 μg/g (S.E. = standard error of mean ± 0·0517). The spleens from 11 cats in which the splenic nerves had been divided at the coeliac axis had a mean acetylcholine equivalent of 0·105 μg/g (S.E. ± 0·0259); the individual values are given in Table 1. The difference between the mean value of the acetylcholine equivalent in normal and denervated spleens was found to be very highly significant when tested by Student's *t* test ($t = 16·7$; $P < 0·001$). The assays of the reineckate-precipitated material from extracts of the spleens were performed on strips of guinea-pig ileum. Since the method of purification by reineckate-precipitation is specific for choline esters (Bentley & Shaw, 1952), it is unlikely that contractions of the ileum were produced by other pharmacologically active substances. The activity on the ileum has been expressed in terms of acetylcholine, although Banister, Whittaker & Wijesundera (1953) have shown that propionylcholine and possibly another choline ester are present in spleens of another species, the ox.

The noradrenaline content was determined in separate portions of the denervated spleens (Table 1). The mean noradrenaline content of 11 denervated spleens in these experiments was 0·205 μg/g, which is the same as the mean value obtained by Burn & Rand (1959*a*) for 8 denervated

spleens. Normal cat spleens contain 1·13 μg of noradrenaline/g (Burn & Rand, 1959*a*).

After degeneration of the sympathetic fibres the percentage fall in acetylcholine content of denervated spleens was 22 % of the value obtained for the normal spleens; the noradrenaline content of denervated cat spleen was 18 % of normal.

Reserpine treatment causes a depletion of noradrenaline in the spleen (Burn & Rand, 1959*a*), but the acetylcholine content of spleen was not affected. The mean value for acetylcholine content in spleens from four reserpine-treated cats was 0·43 μg/g, which was not significantly different from the mean value of 0·47 μg/g found in the intact spleens from normal cats.

TABLE 1. Acetylcholine and noradrenaline contents of denervated cat spleens

Acetylcholine (μg/g)	Noradrenaline (μg/g)
0·02	0·12
0·02	0·06
0·04	0·037
0·04	0·65
0·08	0·07
0·08	0·12
0·10	0·10
0·12	0·10
0·15	0·10
0·20	0·50
0·30	0·06
Mean 0·105	0·205

In two experiments designed to test whether operation *per se* affected the spleen, the nerves accompanying the superior mesenteric artery were divided. Ten days after this operation the spleens were removed and the noradrenaline and acetylcholine contents were determined. The noradrenaline contents of these two spleens were 0·75 and 1·0 μg/g, and the acetylcholine contents 0·4 and 1·0 μg/g, showing that neither was affected.

The release of acetylcholine on stimulating the splenic nerve

In order to test whether an acetylcholine-like substance was released by splenic nerve stimulation we used spleens from reserpine-treated cats to eliminate interference caused by the release of noradrenaline. The release of a substance which resembles acetylcholine into the splenic perfusate after splenic nerve stimulation was successfully demonstrated in each of five experiments.

In order to reduce the time required for handling the samples the venous effluent was allowed to act directly on an acetylcholine-sensitive preparation by arranging it to superfuse a strip of guinea-pig ileum. Stimulation of the splenic nerve in the perfused reserpinized spleen was without effect

on the superfused ileum in the absence of an anticholinesterase. When neostigmine (1 μg/ml.) was added to the fluid perfusing the spleen the ileum contracted, but subsequently relaxed. After neostigmine had been present in the perfusion fluid for 30–60 min, splenic nerve stimulation produced a contraction of the superfused ileum, which started 1–3 min after the beginning of stimulation. Thus, in the experiment shown in Fig. 1, the splenic nerve was stimulated for 1 min at 50/sec; during the period of stimulation there was an increase in the rhythm of the ileum and then a gradual increase in tone leading to a contraction which began about 90 sec

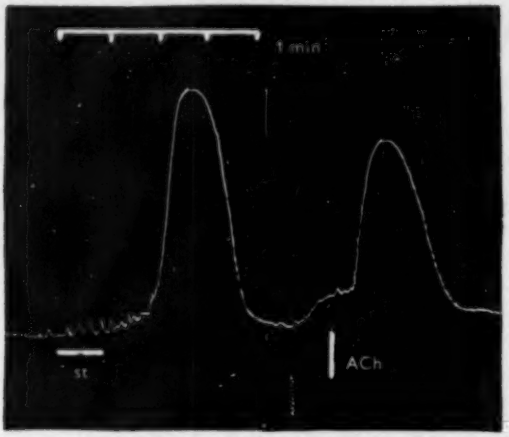

Fig. 1. Contractions of guinea-pig ileum superfused with outflow from the isolated spleen of a reserpinized cat. The spleen was perfused with Tyrode solution containing neostigmine (1 μg/ml.). The splenic nerve was stimulated with 2 msec pulses at 50/sec for 1 min at st. At ACh, 0·2 μg of acetylcholine was injected into the splenic arterial cannula.

after the end of stimulation and was followed by a relaxation which was complete 3½ min after stimulation. The contraction and subsequent relaxation of the ileum were similar in time course to the effects of 0·2 μg acetylcholine injected into the splenic artery cannula. The superfused ileum was contracted by 0·01 ng of acetylcholine when this was injected directly into the superfusion stream as it emerged from the spleen. The relatively weak action of a much larger amount of acetylcholine administered to the ileum via the perfused spleen could be explained either by an incomplete neostigmine block of splenic cholinesterase, with consequent destruction of acetylcholine, or by dilution of the acetylcholine when injected into the spleen. Consequently it is possible that the contraction of the ileum produced by splenic nerve stimulation was due only to a small fraction of the choline esters liberated by these nerves in the spleen.

The contractions of the superfused ileum produced by splenic nerve stimulation or by acetylcholine were abolished by atropine (0·1 μg/ml.) added to the perfusion fluid, as shown in Fig. 2. In this figure four consecutive contractions of the superfused ileum produced by repeated splenic nerve stimulations were followed by a contraction caused by 0·5 μg of acetylcholine injected into the splenic arterial cannula; when 0·1 μg/ml. of atropine was added to the perfusion fluid splenic nerve stimulation was without effect on the ileum.

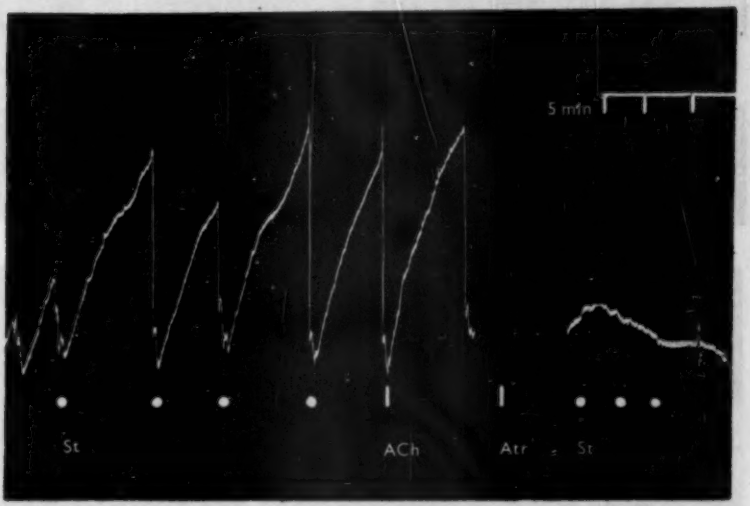

Fig. 2. Contractions of guinea-pig ileum superfused with outflow from a reserpinized cat spleen perfused with Tyrode solution containing neostigmine (1 μg/ml.). The splenic nerves were stimulated with 2 msec pulses at 25/sec for 1 min at the white dots (St). Acetylcholine (0·5 μg) was injected into the arterial cannula at Ach. At Atr, atropine (0·1 μg/ml.) added to the perfusion fluid abolished the contractions of the ileum following splenic nerve stimulation.

Reduction of the response to sympathetic nerve stimulation by hemicholinium

Hemicholinium (50 μg/ml.) added to the perfusion fluid reduced the contractions of the normal spleen in response to splenic nerve stimulation. Thus, in an experiment in which we recorded longitudinal contractions of the isolated perfused spleen produced by sympathetic nerve stimulation for 1 min periods in every 10 min, the contractions observed after perfusing with hemicholinium gradually decreased in size. After 75 min the contraction was 65 % of the original, after 145 min it was 56 % and after 215 min it was 35 %. At this stage choline chloride (500 μg/ml.) added to the perfusion fluid restored the response to 75 % of its original height.

In other experiments the contractions of the spleen produced by sympathetic nerve stimulation were observed by recording the outflow from the spleen, measured with a Thorp drop counter (Fig. 3). When the

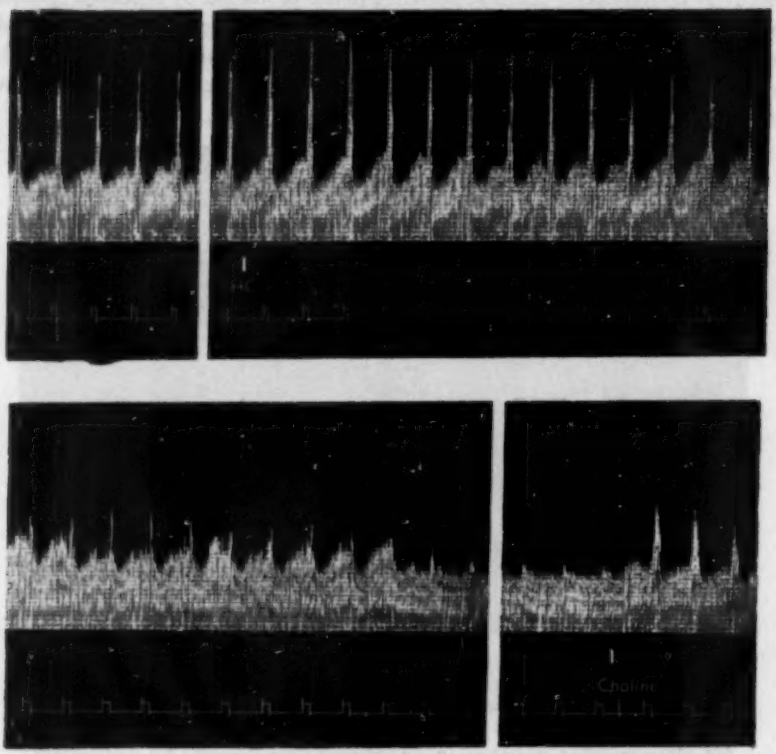

Fig. 3. Contractions of cat spleen in response to splenic nerve stimulation measured by recording the outflow with a drop counter, returning to the base line every 10 sec. Lower signal, stimulation of splenic nerve with 2 msec pulses at 50/sec for 30 sec periods every 4·5 min in *a* and *b*, and for 50 sec periods in *c* and *d*. Initially, in *a*, responses were constant. In *b*, hemicholinium (50 μg/ml.) was added to the perfusion fluid at HC. The interval between *b* and *c* was 200 min, and between *c* and *d* 63 min. In *d* choline chloride (500 μg/ml.) was added to the perfusion fluid and restored contractions.

splenic nerves were stimulated the outflow increased from 16 to 40 drops/10 sec. It was observed that the increase in outflow was due to a visible contraction of the spleen. After the period of stimulation there was a reduction in outflow which accompanied relaxation of the spleen. The addition of hemicholinium (Fig. 3*b*) to the perfusion fluid produced a

transient increase in the responses to sympathetic stimulation and then a gradual decrease leading to complete block (Fig. 3*c*). After the addition of choline to the perfusion fluid there was a partial restoration of the response to stimulation (Fig. 3*d*).

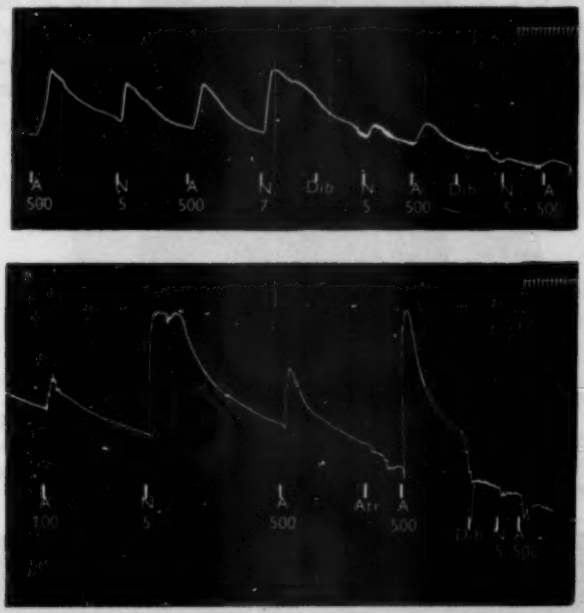

Fig. 4. Longitudinal contractions of isolated perfused cat spleen. Drugs were injected into the arterial cannula. At A (acetylcholine), and at N (noradrenaline), the numerals refer to doses in μg. At Dib, 0·5 mg of dibenzyline (phenoxybenzamine), and at Atr, 0·1 mg of atropine. Records *a* and *b* are from different experiments. Time marker, 30 sec.

Effect of acetylcholine on the spleen

Observations were made on the effect of injections of acetylcholine into the arterial cannula in experiments on the isolated perfused spleen. Figure 4 shows that acetylcholine had about 1/100 of the potency of noradrenaline in producing contractions of the spleen. After the injection of the antiadrenaline drug phenoxybenzamine (dibenzyline) into the cannula the contractions produced by acetylcholine and the contractions produced by noradrenaline were both abolished (Fig. 4). After atropine the injection of acetylcholine caused a bigger contraction than it had before (Fig. 4*b*). Burn & Rand (1960*c*) found that acetylcholine caused a relaxation of the spleen of a reserpine-treated cat and this effect was blocked by

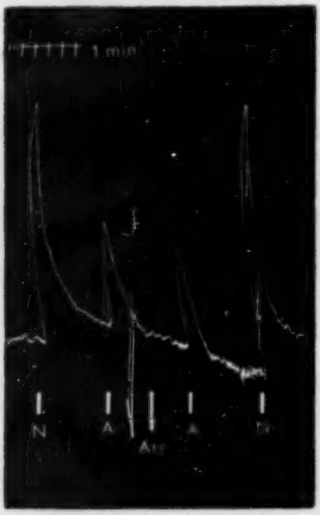

Fig. 5. Contractions of isolated strip of cat spleen in 10 ml. bath. At N, 2 μg noradrenaline, and at A, 200 μg acetylcholine, both washed out after 30 sec. After Atr the bath fluid contained 0·5 μg/ml. of atropine, which did not affect responses to acetylcholine or to noradrenaline.

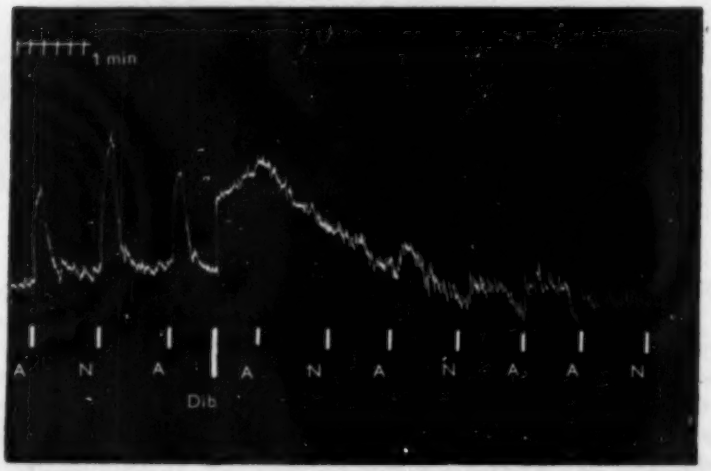

Fig. 6. Isolated cat spleen strip; 10 ml. bath. At A, 300 μg acetylcholine and at N, 20 μg noradrenaline in contact for 30 sec. The spleen strip was exposed to dibenzyline (phenoxybenzamine) 0·1 mg/ml. at Dib for 10 min, which blocked responses to acetylcholine and to noradrenaline.

atropine, which suggests that the larger contraction produced by acetylcholine after atropine in Fig. 4*b* may have been a result of a block of a possible relaxing action of acetylcholine.

Further experiments were carried out on strips cut from spleens and suspended in the organ bath to record contractions. The contractions caused by acetylcholine were not affected by atropine (Fig. 5), but they were abolished by phenoxybenzamine (Fig. 6) and by another anti-adrenaline drug piperoxan (0·2 mg/ml.), as were the contractions produced

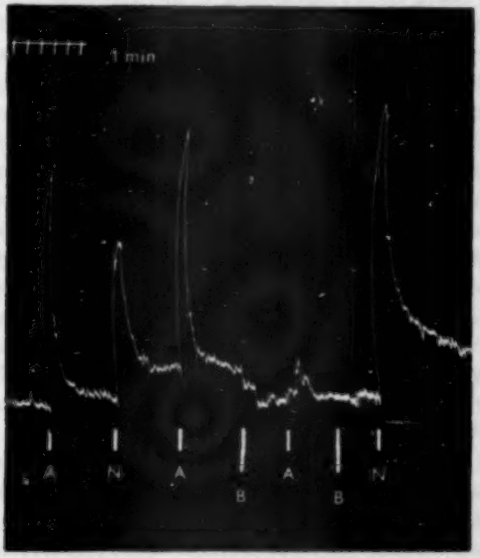

Fig. 7. Isolated cat spleen strip. A, 100 μg acetylcholine; N, 30 μg noradrenaline. At B, 10 μg/ml. of bretylium was added to the 10 ml. bath and washed out after the subsequent responses to acetylcholine and noradrenaline. The response to acetylcholine was blocked by bretylium, whereas the response to noradrenaline was potentiated.

by noradrenaline itself. Bretylium (10 μg/ml.), which abolishes the response to sympathetic nerve stimulation, blocked the contraction of the spleen strip to acetylcholine but the response to noradrenaline was potentiated (Fig. 7).

Spleen strips taken from reserpine-treated cats contracted to a less extent to acetylcholine than did strips from normal cats, whereas the response to noradrenaline was enhanced. After the spleen was exposed to noradrenaline, the response to acetylcholine was increased, whereas the response to noradrenaline was decreased.

DISCUSSION

Acetylcholine has been found in the spleen of the ox and of the horse (Dale & Dudley, 1929; Chang & Gaddum, 1933). Bannister *et al.* (1953) found that three choline esters were present in ox spleen, namely acetylcholine, propionylcholine and a third unidentified ester. We have now shown that cat's spleen contains an acetylcholine-like substance the amount of which is decreased after degeneration of the sympathetic nerves. This decrease could be accounted for if the acetylcholine-like substance were normally contained in the splenic nerve fibres. Acetylcholine has been demonstrated in sympathetic post-ganglionic nerves by Loewi & Hellauer (1938), who found 0·6–2·5 μg/g of acetylcholine in the post-ganglionic fibres from the superior cervical ganglion of cattle. Moreover, the enzymes concerned with acetylcholine metabolism are also present in sympathetic post-ganglionic neurones; Bullock, Grundfest, Nachmansohn & Rothenberg (1947) found that the post-ganglionic fibres from the superior cervical ganglion of the cat contained cholinesterase, and Koelle (1955) has reported that all 'adrenergic' neurones contained variable concentrations of specific acetylcholinesterase. However, there is the possibility that the acetylcholine which disappeared from the spleen on denervation may have originated from preganglionic sympathetic fibres relaying in aberrant sympathetic ganglion cells within or outside the spleen. The histological evidence relevant to this is that Utterback (1944) could not find ganglion cells in the cat's spleen, but Kuntz & Jacobs (1955) have found that some preganglionic fibres were present in the splenic nerve trunk.

Peart (1949) showed that noradrenaline was released from the spleen when the splenic nerves were stimulated. Our experiments demonstrate that in the reserpinized spleen stimulation of the splenic nerves releases an acetylcholine-like substance which contracts the guinea-pig ileum. This substance appeared only after the inhibition of cholinesterase by neostigmine, and its action on the ileum was blocked by atropine; thus it had the properties of a choline ester. If the presence of cholinergic sympathetic fibres to the spleen be accepted, the question must arise, to what extent do the responses to nerve stimulation depend on such fibres? Hemicholinium abolishes contractions of the spleen to nerve stimulation, and previous work has shown that it produces block at cholinergic junctions. For example, hemicholinium can abolish the responses to motor nerve stimulation (Wilson & Long, 1959; Reitzel & Long, 1959*b*), to parasympathetic nerve stimulation (Wilson & Long, 1959) and to preganglionic sympathetic stimulation (MacIntosh *et al.* 1956). MacIntosh *et al.* (1956) showed that hemicholinium inhibited the synthesis of acetylcholine by nervous tissue and they considered that its neuronal blocking action was

due to a gradual disappearance of stored acetylcholine. Choline restored the synthesis of acetylcholine (MacIntosh *et al.* 1956) and thus reversed the failure of transmission at cholinergic junctions (MacIntosh *et al.* 1956; Reitzel & Long, 1959*a*). We have now found that choline restores contractions of the spleen to nerve stimulation after they are blocked by hemicholinium. This suggests that there is a cholinergic junction in the splenic nerves.

Burn & Rand (1959*b*, 1960*c*, *d*) suggested that the role of acetylcholine liberated from post-ganglionic sympathetic fibres may be to release noradrenaline from stores in the tissue, thus rendering the action of these fibres ultimately adrenergic. Acetylcholine, like splenic nerve stimulation, contracts the spleen of the dog (Daly & Scott, 1961) and of the cat. Actions of acetylcholine resembling the adrenergic effects of sympathetic nerve stimulation have frequently been observed, and in many cases it is unlikely that these effects were due to stimulation of sympathetic ganglion cells (Burn, Leach, Rand & Thompson, 1959; Lee & Shideman, 1959). Utterback (1944) has shown that ganglion cells are not present in the cat spleen, although Kuntz & Jacobs (1955) found a few small ganglia in the plexus of the splenic nerve about the splenic artery. A store of noradrenaline in the tissues is necessary for acetylcholine to exert a sympathomimetic action (Burn *et al.* 1959); when the store of noradrenaline is depleted by reserpine this action is lost. We have found that reserpine treatment considerably reduced the action of acetylcholine on the spleen. The stores of noradrenaline in reserpine-treated tissues can be replenished by an infusion of noradrenaline (Pennefather & Rand, 1960). Burn & Rand (1960*c*) found that an infusion of noradrenaline produced an increase in the contractions of the spleen in reserpine-treated cats in response to splenic nerve stimulation, and we have now found that after strips of reserpine-treated spleen were soaked in noradrenaline there was an increase in the response to acetylcholine. Bretylium blocks the response of the spleen to splenic nerve stimulation (Boura & Green, 1959) and we have found that it also blocked the contractions produced by acetylcholine, although the response to noradrenaline was enhanced. The locus of action of bretylium is still uncertain; Boura & Green have concluded that it paralyses sympathetic nerve endings, whereas Burn & Rand (1960*c*) have suggested that it blocks the release of noradrenaline from its peripheral stores by acetylcholine. The adrenergic blocking drugs dibenzyline and piperoxan also blocked the contractions produced by acetylcholine and by noradrenaline. Their action is exerted on the receptors for noradrenaline and the block of the acetylcholine effect is further evidence that acetylcholine was acting through noradrenaline release (cf. Burn & Dutta, 1948). Daly & Scott (1961) have studied the effect of acetylcholine on the isolated blood-perfused spleen

of the dog. They found that large doses of acetylcholine produced contraction of the dog's spleen and, as in our experiments, this response was not blocked by atropine, but was abolished by dibenamine and dibenzyline, and by chronic denervation, and was reduced by reserpine treatment.

Dale (1933) proposed the terms 'adrenergic' and 'cholinergic' to describe those nerves which released (nor)adrenaline and acetylcholine after stimulation. Von Euler (1956) has summarized the evidence that led to the hypothesis that sympathetic adrenergic nerve fibres contain noradrenaline; this evidence is based principally on the findings that sympathetically innervated organs contain extractable noradrenaline, but after section and degeneration of the post-ganglionic sympathetic nerves the noradrenaline content of these organs is diminished. Our findings indicate that sympathetic denervation leads to a loss of the acetylcholine content of the spleen, that sympathetic stimulation releases acetylcholine from the spleen, and that acetylcholine can produce a sympathomimetic effect on the spleen provided that the noradrenaline store is present. Recently Burn & Rand (1960*c*, *d*) have pointed out that there are many accounts in the literature of the occurrence of chromaffin cells in sympathetic nerve trunks and ganglia and in sympathetically innervated organs, although it is not known at present whether there are chromaffin cells in the spleen. These cells, rather than the nerve fibres themselves, could be the site of noradrenaline storage. The suggestion that the chromaffin cells are the site of the noradrenaline store is compatible with the loss of noradrenaline after nerve degeneration seen in some organs, because Burn *et al.* (1959) observed that the chromaffin cells in the nictitating membrane became pyknotic and were reduced in number after denervation. It may be that innervation has a trophic influence in maintaining the integrity of the chromaffin cells or of their noradrenaline content.

SUMMARY

1. The mean acetylcholine content of the cat's spleen was 0·47 μg/g. Ten days after section of the splenic nerve it was 0·10 μg/g. The percentage fall in the acetylcholine content of the spleen which was produced by denervation (78%) is approximately equal to the percentage fall in noradrenaline content after denervation (82%).

2. In reserpinized cats stimulation of the splenic nerves to the isolated spleen perfused with a neostigmine-containing solution led to the release of an acetylcholine-like substance into the venous effluent. This substance contracted the superfused guinea-pig ileum; its action was blocked by atropine.

3. Hemicholinium produced a failure of the contractions of the isolated

cat's spleen in response to nerve stimulation. This effect of hemicholinium was reversed by choline.

4. Acetylcholine had a sympathomimetic action in contracting the isolated spleen. This action of acetylcholine was not blocked by atropine; it was reduced by reserpine treatment, and was abolished by the anti-adrenaline drugs phenoxybenzamine and piperoxan and by the sympathetic nerve-blocking drug bretylium.

5. These findings are discussed in relation to the hypothesis that the sympathetic fibres are cholinergic, and that the acetylcholine then liberates noradrenaline.

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REFERENCES

ARMIN, J., GRANT, R. T., THOMPSON, R. H. S. & TICKNER, A. (1953). An explanation for the heightened vascular reactivity of the denervated rabbit's ear. *J. Physiol.* **121**, 603–622.

BANISTER, J., WHITTAKER, V. P. & WIJESUNDERA, S. (1953). The occurrence of homologues of acetylcholine in ox spleen. *J. Physiol.* **121**, 55–71.

BENTLEY, G. A. & SHAW, F. H. (1952). The separation and assay of acetylcholine in tissue extracts. *J. Pharmacol.* **106**, 193–199.

BOURA, A. L. A. & GREEN, A. F. (1959). The actions of bretylium: adrenergic neurone blocking and other effects. *Brit. J. Pharmacol.* **14**, 536–548.

BÜLBRING, E. & BURN, J. H. (1935). The sympathetic dilator fibres in the muscles of the cat and dog. *J. Physiol.* **83**, 483–501.

BULLOCK, T. H., GRUNDFEST, H., NACHMANSOHN, D. & ROTHENBERG, M. A. (1947). Generality of the role of acetylcholine in nerve and muscle conduction. *J. Neurophysiol.* **10**, 9–21.

BURN, J. H. & DUTTA, N. K. (1948). The action of antagonists of acetylcholine on vessels of the rabbit's ear. *Brit. J. Pharmacol.* **3**, 354–361.

BURN, J. H., LEACH, E. H., RAND, M. J. & THOMPSON, J. W. (1959). Peripheral effects of nicotine and acetylcholine resembling those of sympathetic stimulation. *J. Physiol.* **148**, 332–352.

BURN, J. H. & RAND, M. J. (1959*a*). The cause of the supersensitivity of smooth muscle to noradrenaline after sympathetic degeneration. *J. Physiol.* **147**, 135–143.

BURN, J. H. & RAND, M. J. (1959*b*). Sympathetic postganglionic mechanism. *Nature, Lond.*, **184**, 163–165.

BURN, J. H. & RAND, M. J. (1960*a*). The relation of circulating noradrenaline to the effect of sympathetic stimulation. *J. Physiol.* **150**, 295–305.

BURN, J. H. & RAND, M. J. (1960*b*). The effect of precursors of noradrenaline on the response to tyramine and sympathetic stimulation. *Brit. J. Pharmacol.* **15**, 47–55.

BURN, J. H. & RAND, M. J. (1960*c*). Sympathetic postganglionic cholinergic fibres. *Brit. J. Pharmacol.* **15**, 56–66.

BURN, J. H. & RAND, M. J. (1960*d*). New observations on the sympathetic postganglionic mechanism. *Amer. J. Med.* **29**, 1002–1007.

CHANG, H. C. & GADDUM, J. H. (1933). Choline esters in tissue extracts. *J. Physiol.* **79**, 255–285.

DALE, H. H. (1933). Nomenclature of fibres in the autonomic system and their effects. *J. Physiol.* **80**, 10–11.

DALE, H. H. & DUDLEY, H. W. (1929). The presence of histamine and acetylcholine in the spleen of the ox and the horse. *J. Physiol.* **68**, 97–123.

DALE, H. H. & FELDBERG, W. (1934). The chemical transmission of secretory impulses to the sweat glands of the cat. *J. Physiol.* **82**, 121–128.

DALY, M. DE B. & SCOTT, M. J. (1961). The effects of acetylcholine on the volume and vascular resistance of the dog's spleen. *J. Physiol.* **156**, 246–259.

GADDUM, J. H. (1953). The technique of superfusion. *Brit. J. Pharmacol.* **8**, 321–326.

GARDINER, J. E. (1957). Experiments on the site of action of a choline acetylase inhibitor. *J. Physiol.* **138**, 13–14*P*.

KOELLE, G. B. (1955). The histochemical identification of acetylcholinesterase in cholinergic, adrenergic and sensory neurons. *J. Pharmacol.* **114**, 167–184.

KUNTZ, A. & JACOBS, M. W. (1955). Components of periarterial extensions of celiac and mesenteric plexuses. *Anat. Rec.* **123**, 509–520.

LEE, W. C. & SHIDEMAN, F. E. (1959). Mechanism of the positive inotropic response to certain ganglionic stimulants. *J. Pharmacol.* **126**, 239–249.

LOEWI, O. & HELLAUER, H. (1938). Über das Acetylcholin in peripheren Nerven. *Pflüg. Arch. ges. Physiol.* **240**, 769–775.

MACINTOSH, F. C., BIRKS, R. I. & SASTRY, P. B. (1956). Pharmacological inhibition of acetylcholine synthesis. *Nature, Lond.*, **178**, 1181.

MCEWEN, L. M. (1956). The effect on the isolated rabbit heart of vagal stimulation and its modification by cocaine, hexamethonium and ouabain. *J. Physiol.* **131**, 678–689.

PEART, W. S. (1949). The nature of splenic sympathin. *J. Physiol.* **108**, 491–501.

PENNEFATHER, J. N. & RAND, M. J. (1960). The increase in the content of noradrenaline in tissues after the infusion of noradrenaline, dopamine and L-DOPA. *J. Physiol.* **154**, 277–287.

REITZEL, N. L. & LONG, J. P. (1959*a*). Hemicholinium antagonism by choline analogues. *J. Pharmacol.* **127**, 15–21.

REITZEL, N. L. & LONG, J. P. (1959*b*). The neuromuscular blocking properties of α, α′ dimethylethanolamino 4,4′ biacetophenone (hemicholinium). *Arch. int. Pharmacodyn.* **119**, 20–30.

SHAW, F. H. (1938). Estimation of choline and acetylcholine. *Biochem. J.* **32**, 1002–1007.

THORP, R. H. (1948). A simple recording impulse counter. *Brit. J. Pharmacol.* **3**, 271–272.

UTTERBACK, R. A. (1944). The innervation of the spleen. *J. comp. Neurol.* **81**, 55–68.

VON EULER, U. S. (1956). *Noradrenaline*. Springfield, Illinois: C. C. Thomas.

VON EULER, U. S. & PURKHOLD, A. (1951). Sympathetic denervation and noradrenaline and adrenaline content of spleen, kidney and salivary glands. *Acta physiol. scand.* **24**, 212–217.

WILSON, H. & LONG, J. P. (1959). The effect of hemicholinium (HC-3) at various peripheral cholinergic transmitting sites. *Arch. int. Pharmacodyn.* **120**, 343–352.

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ELECTROMYOGRAPHY OF MUSCLES OF POSTURE: POSTERIOR VERTEBRAL MUSCLES IN MALES

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(*Received* 21 *October* 1960)

Previous investigations (Joseph, Nightingale & Williams, 1955; Joseph & Williams, 1957) have shown that a standardized posture such as the standing-at-ease position can be maintained as far as the lower limbs are concerned by the calf muscles and the ligaments of the knee and hip joints. Consideration of the relation of the line of weight to these joints, i.e. the perpendicular through the centre of gravity of the body, and the structure of the joints themselves can explain the mechanism for maintaining this posture. The vertebral column, however, with its curves, segmental structure and many muscles presents a much more complicated arrangement of joints, and it seemed possible that there would be differences in the activity of the muscles at various levels of the back. It was decided to investigate the posterior vertebral muscles at twelve levels between the upper part of the sacrum and the spine of the fourth cervical vertebra.

METHODS

The double-channel amplifier used was similar to that described previously (Joseph & Williams, 1957). It had a low noise level of 2 μV, so that the minimum detectable potentials were of the order of 3 μV peak-to-peak and had constant gain between 20 and 200 c/s, the response being reduced to one half at 4 and 700 c/s. The apparatus and subject were inside a screening cage in order to reduce interference. Surface electrodes retained in position by suction were used, and the electrode sites were rubbed with Cambridge electrode jelly.

The subjects were 20 males aged 18–46 years, of varied height and weight, with medical histories which suggested no abnormality of their locomotor system. They wore shoes and bathing trunks during the investigation. The position of the spine of every alternate vertebra from the fourth cervical to the second sacral was marked on the skin and the subject was asked to take up the standard posture (standing-at-ease position) which was used throughout the experiment. The electrodes were placed horizontally over the vertebral muscles at the level of the marked spines, one pair on either side about 2 cm from the mid line, with an interval of about 1 cm between the two electrodes of a pair. Frequently the electrodes were left in the same place for as long as 4–5 min and the oscilloscope was observed during the whole of this period. Typical recordings of what was seen on the screen were

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made at all twelve levels. In addition, the pairs of electrodes were often placed at two different levels of the same side and recordings were made.

The recordings were then examined and an assessment made of the amount of electrical activity shown: less than 5 μV peak-to-peak were graded as 'no activity', 5–25 μV 'slight activity', 25–70 μV 'moderate activity' and more than 70 μV 'marked activity'. Figure 1 shows some typical examples of the differences in activity at various levels of the vertebral column.

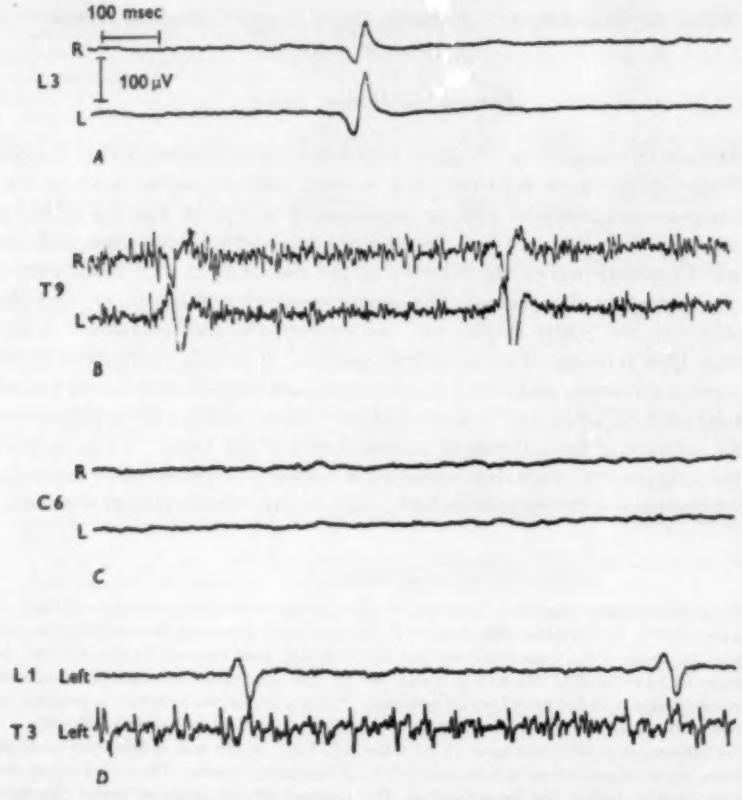

Fig. 1. Recordings from *A*, region of L3; *B*, region of T9; *C*, region of C6; *D*, (above) region of L1, (below) region of T3 of same side to show marked difference in activity at two levels.

RESULTS

Table 1 gives the details of the results (where there was asymmetry in the amount of activity, the greater has been recorded). It can be seen that there is considerable variation between different subjects at any one level. If however the activity in each subject is studied vertically a pattern

emerges in 15 out of the 20 subjects. In the lumbar region, usually at the level of the spine of the third lumbar vertebra, there is little or no detectable electrical activity as compared with the lower thoracic region (the spine of the ninth or eleventh thoracic vertebra). Higher up the detectable activity decreases and no, or only slight, electrical activity is found in the lower cervical region (the spine of the fourth or sixth cervical vertebra).

TABLE 1. Electrical activity* in the posterior vertebral muscles at 12 levels of the vertebral column in 20 subjects while standing at ease

Vertebral level	Subject 1	2	3	4	5	6	7	8	9	10
C4	+	+	++	+	−	++	+	+	−	+
C6	+	++	++	−	+	+++	++	+	++	+
T1	+	++	+++	++	++	+++	++	+	++	++
T3	+	+	+++	++	++	++	++	+	++	++
T5	++	+	−	+++	−	++	++	+	+	+
T7	+++	+++	−	++	−	++	++	++	++	++
T9	+++	+++	−	++	+	++	++	++	++	−
T11	++	+++	+	++	++	++	++	++	++	−
L1	++	+++	+	++	++	++	+	++	−	−
L3	+	+	+	+	−	+	−	+	−	−
L5	+	+	+++	++	−	++	−	+	−	++
S2	+	+++	++	−	+	+++	−	−	+	−
	11	12	13	14	15	16	17	18	19	20
C4	+	+	+	+	−	+	+	+	+	+
C6	+	+	+	+	+	+	+	+	+	+
T1	+	++	+	+	++	+	+	+	+	+
T3	+	++	+	+	−	+	+	+	+	+
T5	+	+	++	+	−	+	+	+	++	+
T7	+	++	++	+	−	++	+++	+	+++	−
T9	+++	++	+++	+++	+	++	+++	+++	+++	−
T11	+++	+	++	−	+	+	++	++	+++	+
L1	+++	+	+	−	+	−	+	++	++	+
L3	++	−	+	−	++	−	+	+	++	+
L5	+	−	+	−	++	+	+	++	+	+
S2	++	+	++	+	+++	+	+++	++	++	++

* − < 5 μV; + 5–25 μV; ++ 25–70 μV; +++ > 70 μV.

DISCUSSION

These results indicate that in the standing-at-ease position the activity of the posterior vertebral muscles varies at different levels. Other workers have usually studied only one region. Åkerblom (1948) found considerable variation between his subjects when he placed the electrodes over the middle of the lumbar region on one side. Floyd & Silver (1955) found little or no activity with the electrodes over 'the most prominent parts of the thoraco-lumbar and lumbar erectores spinae muscles' and Portnoy & Morin (1956) found that 12 out of 16 subjects showed activity in the lumbar region. They, however, placed one electrode at the level of the first lumbar vertebrae and the other at the level of the third. It is not surprising that

they detected some activity in many of their subjects, since the electrodes covered a comparatively wide area.

The differences in muscular activity at various levels may be due to the fact that the line of weight of that part of the body above the level studied lies in front of or behind the transverse axis of the joints between the vertebrae at which flexion or extension is taking place. Examination of a lateral X-ray of the vertebral column indicates that the line of weight of the segment of the body above the lumbar region passes *behind* the transverse axis of the joints between these vertebrae so that the trunk is extending in the lumbar region under the influence of gravity, and the extensors, i.e. the posterior vertebral muscles, are inactive. In the lower thoracic region the reverse is happening and the line of weight passes *in front of* the transverse axis of these vertebral joints. Consequently the posterior vertebral muscles of this region contract and resist flexion of the trunk.

It is more difficult to explain the absence or slight activity of the muscles of the cervical region, since a perpendicular through the centre of gravity of that segment of the body above the seventh cervical vertebra, as determined by Dempster (1955), passes in front of the vertebral bodies and therefore in front of what is probably the transverse axis of movement between adjacent cervical vertebrae. An attempt to find this axis by means of cineradiography and an analyser projector confirmed this. The likeliest explanation is that only slight muscular activity is required to supplement the passive structures (intervertebral disks, ligaments, etc.) which resist flexion of the neck in the standing-at-ease position.

The relatively small amount of activity detected in the posterior vertebral muscles in the lumbar and cervical regions while standing fits in with the observations of Hellebrandt, Brogdon & Teffer (1940) and Booyens & McCance (1957), who found that there was only a small increase in the basal metabolic rate of healthy men and women when standing as compared with the rate when lying down and that any muscular activity increased the rate very considerably.

SUMMARY

1. The activity of the posterior vertebral muscles at twelve levels in 20 males standing at ease has been studied by means of electromyography.

2. Although there is considerable variation in the detectable activity at one level, a pattern of activity in a vertical direction can be seen. There is little or no activity in the lumbar and cervical regions and moderate or marked activity in the lower thoracic.

3. The possible explanation of these observations is discussed.

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REFERENCES

ÅKERBLOM, B. (1948). *Standing and Sitting Posture*, pp. 142–144. Stockholm: A.-B. Lordiska Bokhandelm.

BOOYENS, J. & MCCANCE, R. A. (1957). Individual variations in expenditure of energy. *Lancet*, **273**, 225–229.

DEMPSTER, W. T. (1955). Space requirements of the seated operator: geometrical, kinematic and mechanical aspects of the body, with special reference to the limbs. *Wright Air Development Center Technical Report*, 55–159, 1–241.

FLOYD, W. F. & SILVER, P. H. S. (1955). The function of the erectores spinae muscles in certain movements and postures in man. *J. Physiol.* **129**, 184–203.

HELLEBRANDT, F. A., BROGDON, E. & TEPPER, R. H. (1940). Posture and its cost. *Amer. J. Physiol.* **129**, 773–781.

JOSEPH, J., NIGHTINGALE, A. & WILLIAMS, P. L. (1955). A detailed study of the electric potentials recorded over some postural muscles while relaxed and standing. *J. Physiol.* **127**, 617–625.

JOSEPH, J. & WILLIAMS, P. L. (1957). Electromyography of certain hip muscles. *J. Anat., Lond.*, **91**, 286–294.

PORTNOY, H. & MORIN, F. (1956). Electromyographic study of postural muscles in various positions and movements. *Amer. J. Physiol.* **186**, 122–126.

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GLOMERULAR FILTRATION AFTER OBSTRUCTION OF THE URETER

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Since its introduction by Malvin, Wilde & Sullivan in 1958 'stop-flow' analysis has been widely used to localize reabsorptive and secretory processes and the site of action of diuretics in the nephron. The concentration patterns revealed by this method are not always easy to interpret, one of several reasons being that it is uncertain to what extent they are affected by the continued passage of glomerular filtrate into the renal tubules during the period of occlusion. Hungarian workers in particular have long maintained that after ligation of the ureter glomerular filtration continues at one quarter to two thirds the normal rate (Jancsó, 1955), and stop-flow analysis has been condemned as 'useless' on these grounds (Foeldi, Papp & Koltay, 1958).

We have attempted to estimate, by a modification of the original stop-flow procedure (Malvin, Wilde & Sullivan, 1958), how long and at what rate glomerular filtration will continue in the dog's kidney if its ureteric outflow is suddenly obstructed during intense osmotic diuresis. Substances believed to enter the renal tubules only by glomerular filtration were injected intravenously at various times after blocking the ureter. The subsequent changes in plasma concentration were determined, as well as the total amount of these substances appearing when flow recommenced ahead of urine derived from filtrate formed after release of the obstruction. From the data the rate of entry of glomerular filtrate during the period of occlusion was calculated. The distribution of the reference material in the early (or 'distal') and late (or 'proximal') samples was also considered. In addition, the pressure changes in the ureteric catheter above the obstruction were recorded. A few experiments were performed in a somewhat different manner in order to study the effect on glomerular filtration of obstructing the outflow from the kidney at lower rates of urine flow.

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METHODS

Bitches weighing from 10 to 20 kg were used. They were anaesthetized with morphine tartrate 2 mg/kg subcutaneously, followed 40 min later by intravenous injection of a mixture of 50 mg/kg of chloralose (Roche) and 500 mg/kg of urethane dissolved in normal saline. The solution is strongly hypertonic and caused mild osmotic diuresis, with resting urine flows of 0·2–1·0 ml./min.

Through a flank incision the left ureter was cannulated retro-peritoneally with fine plastic tubing, which was advanced until the upper orifice lay 2 or 3 mm below the hilum of the kidney. In some experiments the right ureter was also cannulated, but in the majority the right kidney drained through a self-retaining catheter inserted into the bladder.

All injections were made into the left external jugular vein and slow sustaining infusions entered by the same route. Hypertonic solutions given at fast rates were infused through polythene tubing introduced into the abdominal vena cava via the right femoral vein. Arterial blood samples were taken from the right carotid, and blood pressure was recorded from the right femoral artery. Mercury or capacitance manometers were used to record arterial and ureteric pressures. Heparin (Liquemin, Roche, 100 u./kg approx.) was used as anticoagulant.

The routine followed in all experiments was based on the method of 'stop-flow analysis' described by Malvin, Wilde & Sullivan (1958) but differed from their procedure in various ways according to the type of experiment.

(1) *Experiments to demonstrate filtration during ureteric obstruction.* Diuresis was produced by infusion of 20% mannitol in 0·85% NaCl solution at a constant rate of 8–12 ml./min, depending on the size of the animal. No other chemicals were added to the infusion, except para-aminohippurate (PAH), 0·045 g/100 ml. on three occasions only, after 0·15 g PAH had been given as a priming injection. When the urine flow from the left kidney had reached a steady level exceeding 7 ml./min the ureteric catheter was clamped for 8 min. At the moment of occlusion (zero time in Fig. 1) 1·0 g creatinine in 10 ml. saline was rapidly injected into the jugular vein; 0·7 or 0·8 g inulin in 10 ml. saline was rapidly injected 2 or 4 min after the start of occlusion; and a slow steady injection of 1·0 g sodium thiosulphate in 50 or 100 ml. saline was begun 6 min 45 sec after zero time and completed 15 sec before release of the occlusion. Immediately following this, thirty serial urine fractions of 0·4–1·1 ml. each were collected in 1½–2½ min. From the moment of occlusion onward thirty-eight to forty serial blood samples of *ca.* 2 ml. were taken, every 5 sec during the first half minute following each injection of creatinine or inulin, and at gradually increasing intervals thereafter. The last sample was taken at the end of the collection of urine fractions. Before and after the period of occlusion several timed 'free-flow' urine specimens and corresponding blood samples were collected for clearance determinations.

(2) *Experiments to estimate the rate of residual filtration.* These were similar to those described above except for the following modifications. Diuresis was induced by infusion of 23% mannitol, or alternatively, of 10% mannitol and 15% glucose in normal saline, without addition of other chemicals. If glucose was used a priming injection of 10·0 g dextrose in 25 ml. saline was given before starting the infusion. The ureteric outflow was obstructed for 11 min and the pressure in the ureteric catheter was registered throughout this period by a mercury manometer with minimal fluid displacement. One gram creatinine in 10 ml. saline was rapidly injected exactly 6 min after the start of occlusion and serial blood sampling was begun at the same moment, intervals being staggered as described above. A slow injection of 0·7 or 0·8 g inulin in 50 ml. saline was begun 9 min 45 sec after the start of occlusion and completed 30 sec before release of the obstruction. Thirty fractions were collected in 2 min and the last of twenty-four blood samples was taken at the end of this period.

(3) *Control experiments.* In order to study the stop-flow pattern for creatinine which

developed when plasma creatinine concentration was not changing during the period of ureter obstruction, control experiments were performed. In these a steady raised plasma level was maintained by continuous infusion of creatinine, but no injections were made at the start or during the occlusion period apart from the 'new filtrate marker' (sodium thiosulphate, or inulin) which was given just before the release of the block, as usual. Blood was sampled only during the clearance periods before and following occlusion. For comparison with the first type of experiment another five dogs were given an intravenous priming injection of 0·7 g creatinine in 10 ml. saline, and 0·2 g creatinine/100 ml. was added to the 20% mannitol solution infused subsequently to raise the urine flow. The ureter was clamped for 8 min. Control runs for the second series of experiments described above were performed after completion of the main experimental run on the same animals. The creatinine injected during the experiment proper served as 'priming' injection for the control run. After collection of all experimental samples the infusion fluid was changed to one containing 0·2 g creatinine/100 ml. in addition to mannitol, or mannitol and dextrose, in the same concentration as before. Approximately 15–20 min after the end of the first run the ureter was again clamped for 11 min. Finally, a 'new filtrate' marker was injected and a second set of urine fractions was obtained in the usual manner.

(4) *Experiments to estimate filtration following ureteric obstruction at low rates of urine flow.* Both ureters were cannulated and the simultaneous output of the two kidneys was compared. A priming dose of 1 g creatinine in 25 ml. saline was injected intravenously and a constant plasma level was maintained by a sustaining infusion of a solution of 5% creatinine in saline, given at the rate of 0·35–0·50 ml./min. The left ureter was blocked 30–40 min after the priming injection. The duration of occlusion varied. The right kidney drained freely throughout. Inulin, and on some occasions sodium thiosulphate, was injected slowly starting 75 sec before the end of the occlusion period, to mark filtrate formed after the release of the obstruction. Fifteen to thirty fractional urine samples were collected, the time necessary to obtain adequate volumes varying with the rate of flow. After a brief interval a second experiment was performed in which the duration of occlusion was a third of that in the first run. Blood samples were taken at the mid points of preliminary and final 'free-flow' periods, and at regular intervals of 6 or 10 min during the rest of the time.

Chemical analyses. The volume of urine fractions was determined by weighing. Creatinine in plasma and urine was determined colorimetrically, as the alkaline picrate. Inulin in plasma and urine was estimated by the resorcinol method of Schreiner (1950). If glucose is present this method gives high plasma and urine blanks. By using yeasted samples, and by control occlusions without injection of inulin it was ascertained that despite the high blank the method was adequate for urine analysis; the arrival of the first trace of inulin in the urine samples also was recognizable without difficulty. The method was found not to be sufficiently accurate for determination of inulin in plasma in the presence of glucose and was not used for this purpose. Sodium was determined with an EEL flame photometer. Thiosulphate in urine was determined according to the method described by Gilman, Philips & Koelle (1946). This substance was used infrequently. As only 90 sec or less elapsed between injection of the material and release of the ureter any effective 'shortening' of the occluded fluid column which might have been caused by its being secreted into the tubules was probably negligible.

Calculations

The occluded volume, or renal 'dead space'. The serial urine samples emerging after the release of the ureteric block were of three kinds: samples derived entirely from glomerular filtrate formed *before* or *during* the period of ureteric block; samples derived entirely from 'new' filtrate which had passed into the nephrons after the injection of the final filtrate marker (inulin or thiosulphate); and samples which were mixtures of urine derived from both types of filtrate. The first kind contained none of the final filtrate marker (0–13·8 ml. in Fig. 2); the second kind contained it in the same (or a slightly higher) concentration as the

'free-flow' specimens collected after the fractions (18·4–20·6 ml. in Fig. 2); the third kind contained the marker at lower concentrations (13·4–18·4 ml. in Fig. 2). The part v_n of a mixed fraction of volume v_m which consisted of 'new' filtrate was calculated as

$$v_n = v_m \frac{c_m}{c_M},$$

where c_m = concentration of 'new filtrate' marker in the mixed sample, and
c_M = concentration of 'new filtrate' marker in urine derived wholly from 'new' filtrate.

The residue, $v_o = v_m - v_n = v_m(1 - c_m/c_M)$ was derived from filtrate formed *before* or *during* the period of occlusion. For example, in Fig. 2 sample 24, which brought the accumulated volume to 16 ml., contained 22 m-mole $Na_2S_2O_3$/l. In later samples the concentration rose to a steady value of 50 m-mole/l. Thus the fraction of new filtrate in sample 24 was 22/50 = 0·44. This calculation is valid only if (*a*) the plasma concentration of the final marker remained constant during the relevant period, and (*b*) if all urine fractions containing it were concentrated to the same extent during their passage through the tubules.

During the 2–2½ min spent in collecting thirty urine fractions there was usually a just detectable fall in the plasma concentration of the final filtrate marker. Usually only the last twelve to sixteen urine fractions contained the marker. Because of delay time these must have come from filtrate formed during the first 1–1½ min after release of the block. The decline of plasma concentration during this short interval was negligible. We have no direct evidence on whether or not all fractions were concentrated to the same degree. However, it was improbable that large differences arose within the 60–100 sec between the appearance of the first and last of the fractions containing the marker, since these late fractions, in contrast to the first three to five samples, all emerged at the same rate of flow.

The total 'occluded' volume V_o was the sum of all fractions of urine derived from filtrate formed *before* or *during* ureteric obstruction.

The amount of creatinine and/or inulin excreted in the occluded volume. The total creatinine or inulin content of all fractions consisting wholly or partly of 'occluded' urine was determined, and the amount due to admixture of 'new' (i.e. formed after flow recommenced) filtrate urine subtracted (see Fig. 2). The residue must have entered the tubules before the 'new filtrate' marker was injected.

The admixture of inulin or creatinine due to 'new filtrate' was calculated as $\Sigma v_n . U$, where
v_n = urine derived from 'new' filtrate in the mixed fractions, and
U = concentration of creatinine (or inulin) in the first fraction which was derived wholly from 'new' filtrate.

This was based on the following reasoning: 'New' filtrate contained inulin or creatinine at the concentration existing in plasma at the time of its formation. From analysis of timed blood samples (Fig. 1) it was known that the plasma concentration of the material remained virtually constant during the brief interval in which the 'new' filtrate for all mixed and the early unmixed fractions must have entered the nephrons. As before, it was assumed that all were concentrated to the same degree during their passage through the nephrons. Hence, if the portion v_n of a mixed sample had emerged from the ureter uncontaminated by occluded urine it would have contained creatinine or inulin in the same concentration as the first fraction which was wholly derived from 'new' filtrate.

The total inulin or creatinine present in the total occluded volume was obtained by summation of the amounts present in the 'occluded' fractions, and designated Q.

In the case of creatinine, Q included an unknown quantity q of endogenous creatinine which was already present in the nephrons at the moment of occlusion of the ureter. This could not be measured directly. An estimate of its magnitude was made according to the formula

$$q = 1{\cdot}2 \times V_o \times \frac{U_1}{100},$$

where V_o = total occluded volume, and

U_1 = concentration of endogenous creatinine in 'free-flow' urine collected immediately before the start of occlusion.

The values found indicated that endogenous creatinine accounted for from 1·5 to 9·6% of the total recovered in the 'occluded' urine, according to the type of experiment. This is likely to be an over-estimate rather than the converse, for the following reasons:

(1) The recovered occluded volume V_o was probably larger than the volume of fluid present in the nephrons at the moment of occlusion;

(2) Study of stop-flow patterns for creatinine in control experiments on the same animals during which the plasma creatinine level was maintained at a *steady* raised level showed that the *average* creatinine concentration for all fractions of 'occluded' urine was always less than 1·2 times the concentration in the free-flow specimen collected just before occlusion. The factor 1·2 in the formula above thus represents the upper limit of change. In the same control studies it was also established that no single fractional sample contained creatinine at a concentration as high or higher than twice the free-flow concentration. Hence in those experiments in which creatinine was injected during occlusion, samples were regarded as containing exogenous material if the concentration exceeded 2·5 times the free-flow concentration.

Mean plasma concentration of injected creatinine and/or inulin. The concentrations of the test materials in the serial plasma samples were plotted against time in seconds (Fig. 1). The area below the curve between time 0 (instant of injection of test material) to time t (instant of release of ureteric block) was equal to $\bar{c}t$, $\bar{c}$ being the mean plasma concentration.

Rate of glomerular filtration during occlusion of the ureter. For the purpose of estimating the volume of filtrate which entered the 'occluded' kidney certain assumptions were made which are discussed in the text. If the test materials (e.g. inulin in Figs. 1 and 2) were injected after an interval of 2 min or more had elapsed since the start of ureteric obstruction, the rate of residual filtration, RFR was calculated as follows:

$$\text{RFR} = \frac{Q-q}{\bar{c}t} \times 60 \text{ ml./min} \quad \text{(symbols having the same meaning as before).}$$

This tended to underestimate the true RFR, first because the values of q used may have been too large, as discussed above, and secondly because t was the time in seconds between injection of the test material and release of ureteric block, and not between injection of test material and injection of final filtration marker. An error, for which no correction could be readily applied, must have resulted from the fact that an unknown quantity of the final filtration marker very probably passed through the glomeruli into the tubules during the 75 sec between its first appearance in the blood stream and the release of the ureteric clamp.

In five experiments an estimate was sought of the total volume of filtrate which entered the kidney in the course of 8 min of occlusion. In these experiments, with the time (t) measured in minutes from the beginning of occlusion, creatinine was injected at $t = 0$, and inulin at $t = 2$.

In the first attempts on this problem it was assumed that the filtration rate $F(t)$, declined exponentially to zero from its initial value (F_1) determined during the control clearance period before occlusion. It appeared, however, that on this basis $F(t)$ would be down to about 1% of its initial value at the end of 2 min and the observed excretion of inulin in the subsequent period (see Fig. 2) could not be accounted for. The assumption was therefore made that the filtration did not decline to zero, but to a constant value (F_0), which it attained, for practical purposes, by $t = 2$, i.e.

$$F(t) = F_0 + F_1 e^{-\beta t},$$

which is the general equation of the heavy line in the lower part of Fig. 1. The line thus represents the assumed relation between filtration rate and time. The total filtration during the period of occlusion is given by the area beneath this curve, i.e. by

$$\int_0^8 (F_0 + F_1 e^{-\beta t})\, dt \sim 8F_0 + \frac{F_1}{\beta}.$$

The quantities of F_0, F_1 and β were deduced from the amounts of inulin and creatinine excreted. At any moment, the rate of entry into the occluded volume of either of these substances is given by the product of the filtration rate at that moment and their concentration in the plasma at the same moment; the total quantity in the occluded volume will be the integral of the momentary rates of excretion.

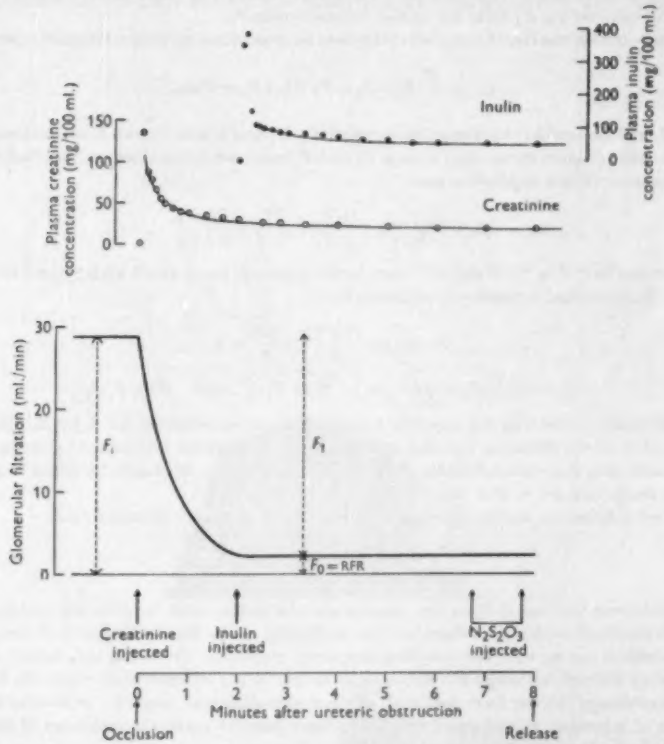

Fig. 1. The period during which the ureter was obstructed in the same experiment as that of Fig. 2. Abscissa, time (t) after ureteric obstruction. The upper curves show the observed concentrations of creatinine and inulin following their intravenous injection at 0 and 2 min respectively.

The thick line shows the assumed course of glomerular filtration during the occlusion period. To calculate total filtration which is the area below this line, it is necessary to know F_0, and how the line falls from F_t (i.e. the exponential β in the text).

The course of the creatinine and inulin concentrations in the plasma subsequent to injection (Fig. 1) was expressed in the forms

$$C(t) = C_0 + C_1 e^{-\alpha t}$$

and

$$D(t) = D_0 + D_1 e^{-\gamma t}, \text{ respectively.}$$

Thus, for inulin

$$Q_I = F_0 \int_2^8 (D_0 + D_1 e^{-\gamma t})\, dt,$$

where Q_I was the amount of inulin excreted, and the terms D_0, D_1 and γ had been determined from the inulin plasma concentration curve (Fig. 1). F_0 was thus obtained, and F_1 was found by subtracting F_0 from the initial filtration rate F_I.

The exponent β was then found, using the data on creatinine, by evaluating the expression:

$$Q_C = \int_0^8 (F_0 + F_1 e^{-\beta t})\,(C_0 + C_1 e^{-\alpha t}) dt,$$

when Q_c was the amount of creatinine excreted, C_0, C_1 and α were known from the creatinine plasma concentration curve (Fig. 1), and F_0 and F_1 had been determined as described above.

Integration of this expression gave

$$Q_C = F_0\left[8C_0 + \frac{C_1}{\alpha}\right] + F_1\left[\frac{C_0}{\beta} + \frac{C_1}{\alpha+\beta}\right],$$

where terms in $e^{-8\beta}$, $e^{-8(\alpha+\beta)}$ and $e^{-8\alpha}$ have been neglected, being small with respect to unity.

One thus obtained a quadratic equation for β

$$\beta^2 - \beta\left[\alpha - \frac{B+G}{A}\right] - \frac{\alpha B}{A} = 0,$$

where $A = [Q_C - F_0(8C_0 + C_1/\alpha)]$, $B = F_1 C_0$ and $G = F_1 C_1$

and the positive root was the quantity sought. The values obtained for β, by this method, were such that the filtration rate did in fact appear to approach a constant value for $t > 2$, thus confirming the reasonableness of the initial assumption. It should be noted that there was no restriction set to the value of β.

The total filtration during the period of occlusion was finally obtained from

$$\int_0^8 (F_0 + F_1 e^{-\beta t})\, dt \sim 8F_0 + \frac{F_1}{\beta}.$$

It is obvious that apart from the assumption of a simple form for $F(t)$, the major simplification involved in this procedure was the neglecting of the short rising limb of the plasma concentration curve; with the sampling frequency employed, this could not, in any case, be accurately defined, although the subsequent course of the concentration could be followed with confidence. In the form assumed, the concentration was taken to be maximal at the instant of injection; the effect of this would have been to make the estimates of filtration slightly lower than the true value.

In some cases the plasma concentration curves were not adequately fitted by the forms given above, and an additional linear trend was incorporated; the method of solution, though somewhat lengthier than that outlined here, was in principle the same.

RESULTS

Persistence of glomerular filtration after obstruction of the ureter

Creatinine injected intravenously immediately after occlusion of the ureter was found after release of the obstruction 8 min later in all serial urine samples, with the occasional exception of the first. Thus 96·3–100 % of the 'occluded' volume was contaminated with material that had crossed the glomerular membrane after the ureter was clamped. One of six

experiments, all of which gave similar results, is illustrated in Fig. 2. The simultaneous concentration patterns for sodium and para-aminohippurate (PAH) are also shown.

When inulin was injected 2 min after clamping the ureter, and allowed to circulate for 6 min before the obstruction was removed, the first 10%

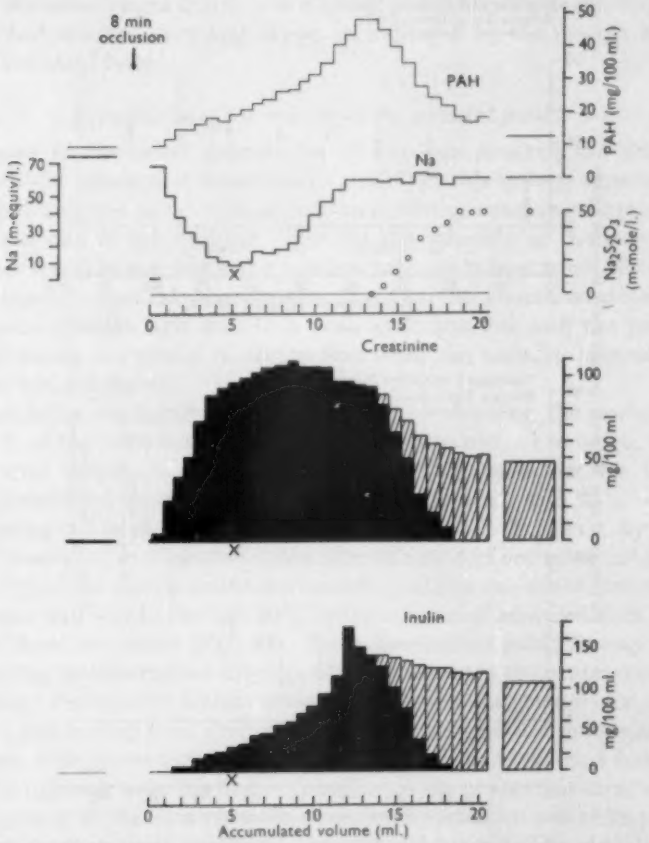

Fig. 2. The creatinine and inulin content of urine collected after 8 min obstruction of the ureter. Infusion of 20% mannitol in saline containing PAH, at 10 ml/min. Left ureter occluded when urine flow on this side was 9·9 ml./min. 1 g creatinine injected I.V. at the time of occlusion; 1 g inulin injected I.V. 2 min after occlusion; 0·9 g sodium thiosulphate injected I.V., starting 6 min 30 sec after occlusion. Top: Stop-flow concentration patterns for sodium, para-amino hippurate and thiosulphate. Centre and bottom: Creatinine and inulin present in the fractions. Black: filtered during ureteric occlusion. Hatched: filtered after release of obstruction.

of the occluded column of fluid to emerge was inulin-free in four experiments; in the remaining two, inulin was recovered from all fractions. In two further experiments, in which inulin was injected at the mid point of an occlusion lasting 8 min, the distal 10 and 27% respectively of the occluded fluid were inulin-free.

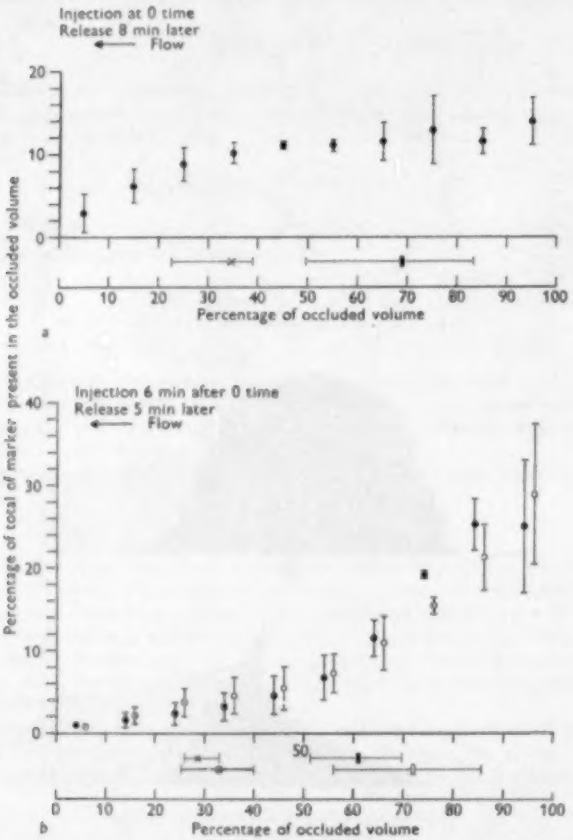

Fig. 3. Distribution in the occluded volume of creatinine injected after occlusion of the ureter. Mean values and standard deviations for groups of six experiments. (*a*) Distribution of creatinine injected at time of occlusion. Infusion fluid, 20% mannitol in saline. (*b*) Distribution of creatinine injected 6 min after start of occlusion. Filled circles, 23% mannitol infused; open circles, 10% mannitol + 15% glucose infused.

Also shown are: the mean position and range of the point of lowest sodium concentration (crosses) and of the first trace of material derived from filtrate formed after release of occlusion (vertical bars).

If injected 6 min after ureteric occlusion and left to circulate for 5 min before releasing the obstruction, exogenous creatinine likewise appeared in most of the serial urine samples, except the most distal fractions which comprised from 3 to 28 % of the occluded volume. These results showed that substances believed to enter the nephrons only by glomerular filtration continued to do so when the outflow from the kidney was blocked, not only in the initial stages during which ureter pressure rose steeply, but also after it had attained very high levels, as indicated by the records which will be discussed later.

Distribution of the marker in the occluded fluid

Contrary to the usual conventions of stop-flow analysis the urinary concentration patterns of creatinine (or inulin) in this type of experiment could obviously not be taken to reflect the extent of water reabsorption at different points of the nephron. Nor did the quantity of the reference substance found in any particular fraction indicate in how much filtrate it was originally contained, because after injection the plasma creatinine or inulin concentration first rose to a peak and then fell, and the precise moment during the period of obstruction when the material entered the nephron was not known.

If creatinine was injected at the moment of clamping the ureter the first 30 % of the occluded volume to emerge contained, on average, 18 % of the total exogenous creatinine recovered. The remainder was fairly evenly distributed through the rest of the 'dead space', with 25 % of the total coming out in the last 20 % of the occluded fluid (Fig. 3*a*). By contrast, if creatinine was injected 6 min after the start of occlusion not more than 5–7 % of the total found in the occluded volume was in the first 30 %, and almost half was in the last 20 % of the column of occluded fluid as it emerged from the ureter (Fig. 3*b*). Both distribution patterns may give a misleading picture in that they appear to exaggerate the contamination of the distal fractions by filtrate entering during the obstruction compared with samples coming from proximal sites. It is perhaps not unreasonable to assume that the molecules of the marker which had travelled farthest down the nephron were the first to pass across the glomerular membrane after injection, at the time when the plasma concentration was at its peak. If this was so the small quantities of marker which reached the distal sites probably represented very small volumes of filtrate. It follows that of a non-reabsorbable substance present in *constant* concentration in filtrate formed after ureteric obstruction only a small quantity would reach the distal segment, whereas much greater amounts would continue to pass into the proximal third of the occluded tubules.

In one experiment out of six the distribution in the 'dead space' of

inulin injected 2 min after clamping the ureter resembled that of creatinine injected at the moment of occlusion (Fig. 3*a*); in three experiments it resembled that of creatinine injected 6 min after blocking the outflow (Fig. 3*b*); and in the remaining two it was intermediate between these

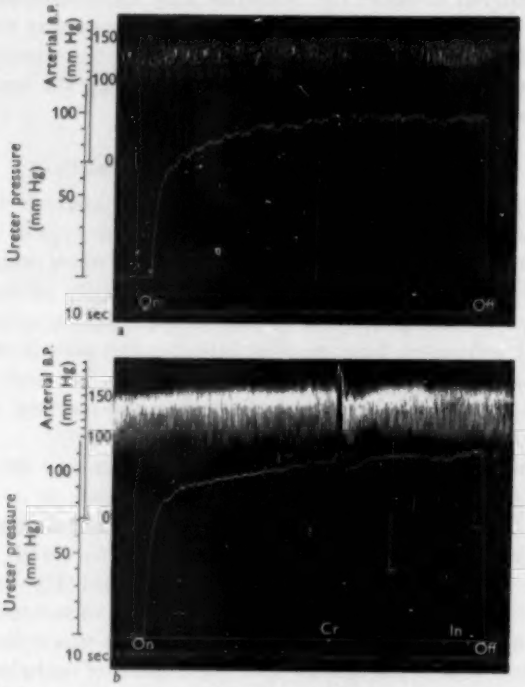

Fig. 4. Typical pressure records obtained from the obstructed ureter (two dogs). In each record, upper trace: femoral arterial pressure, lower trace: ureter pressure. In both experiments 23% mannitol was continuously infused at 10 ml./min, and the ureter was occluded for 11 min.

(*a*) Initial urine flow from left kidney 12·2 ml./min. No injections were made during occlusion.

(*b*) Initial urine flow from left kidney 11·8 ml./min. 6 min after start of occlusion rapid I.V. injection of 1 g creatinine (Cr). On this occasion this caused a transient rise of arterial B.P., which was reflected in the ureter pressure. Final slow injection of 0·7 g inulin (In) caused no disturbance of blood pressure, but a slight rise of ureter pressure.

types. This suggested that the initial phase during which filtrate apparently could still enter rapidly was almost over at the end of 2 min as is shown in the curve in Fig. 1. A change of conditions at about this time was also suggested by the records of ureter pressure.

The pressure in the obstructed ureter

Typical records of ureter pressure are reproduced in Fig. 4. In these tracings four characteristic stages could usually be distinguished. In the first 10–40 sec the pressure above the obstruction rose rapidly to levels ranging from 55 to 95 mm Hg. During the next 30–90 sec the pressure curve became inflected. This was followed by a slow steady pressure rise, with the slope of the curve remaining constant for several minutes. In the

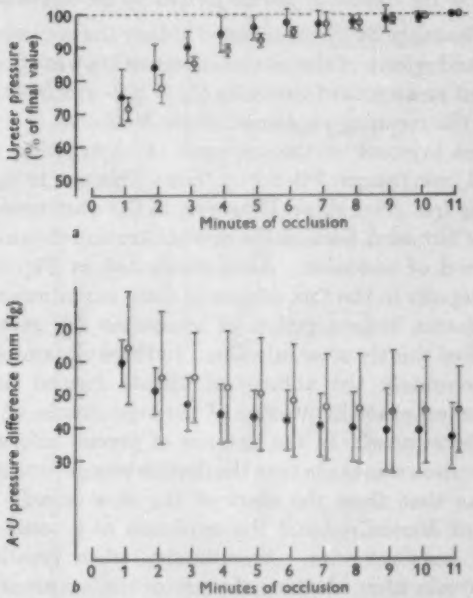

Fig. 5. (*a*) Ureter pressure after occlusion expressed as a percentage of the value attained immediately before release of the obstruction. Mean values and standard deviations. Filled circles, experiments without glucose; open circles, experiments with glucose in infusion fluid. (*b*) *A–U* pressure differences (femoral artery–ureter). Mean values and standard deviations. Same experiments as in (*a*).

final phase the rise of pressure virtually ceased and the curve flattened out. This rarely occurred before the end of the fifth minute of occlusion, and in some experiments this last stage was not reached at all. Any alteration of the mean arterial pressure, whether sudden or gradual, was reflected in the ureter pressure by a change in the same direction but of lesser magnitude. A small upward step in pressure frequently followed upon the injection of each filtration marker.

In Fig. 5 the average minute-to-minute changes in ureter pressure are

expressed as percentage of the value reached at the end of 11 min of ureteric obstruction. The latter ranged from 75 to 148 mm Hg. The final difference between femoral arterial and ureter pressure ranged from 26 to 73 mm Hg. Figure 5 shows that 2 min after occlusion ureter pressure averaged about 81 % and 6 min after occlusion about 95 % of the final height attained. These percentages would have been slightly higher if the terminal injection of inulin or thiosulphate had not been made.

Estimation of the volume of filtrate formed by the obstructed kidney

The plasma clearance by the obstructed kidney in t sec between injection of the material and release of the ureteric obstruction was $C_t = (Q-q)/\bar{c}$ ml; and the apparent mean rate of clearance $C_{\overline{m}} = (Q-q) \times 60/\bar{c}t$ ml./min (each symbol having the meaning explained under Methods).

For creatinine injected at the moment of obstructing the ureter $C_{\overline{m}}$ averaged 4·7 ml./min (range: 3·0–6·2 ml./min). This was 18 % of the plasma clearance during free urine flow. However, in the obstructed kidney $C_{\overline{m}}$ is not a measure of filtration, because the rate of filtration obviously diminishes during the period of occlusion. As is illustrated in Fig. 1, it probably dropped most rapidly in the first minute in these experiments, at the same time as the plasma concentration of creatinine fell steeply from the maximum reached shortly after injection. In these circumstances, in order to calculate accurately the volume of filtrate formed during a given interval of time, an exact knowledge of the time course of the decline in filtration rate is required. In the absence of precise information on this point the assumption was made that the decline was exponential. A further assumption was that from the start of the slow steady rise in ureter pressure onward filtrate entered the nephrons at a much reduced, but approximately constant rate. Accordingly, when creatinine was not injected until 6 min after blocking the ureter the apparent mean plasma clearance $C_{\overline{m}}$ was taken to equal the 'residual' filtration rate (RFR). It must be stressed, however, that the values presented in what follows are to be regarded as estimates rather than precise measurements, and that they depend on premises whose validity has yet to be established.

Calculations based on the foregoing assumptions (see Methods) indicated that from the beginning of the occlusion to the moment of release 8 min later a total of 12–25 ml. of glomerular filtrate entered the obstructed kidney. This was 5·6–10·8 % of the total volume of filtrate that would have been formed in the same time in conditions of free urine flow (Table 1).

If these calculations are valid it follows that the volume of filtrate formed during ureteral obstruction was from 1·3 to 2·2 times larger than the occluded volume. The free-flow creatinine U/P ratios in these experiments ranged from 2·46 to 3·87. Control experiments carried out in comparable

TABLE 1. Mean plasma clearances and estimated total filtration during 8 min of ureteric obstruction. Creatinine injected at instant of blocking ureter; inulin injected 2 min later

		Free flow			Ureteric obstruction						
						Creatinine		Inulin		Estimated total volume filtered	
No.	Body weight (kg)	Urine flow (ml./min)	GFR (ml./min)	U/P creatinine	Occluded volume (ml.)	C_f (ml.) $\frac{Q-q}{c}$	$C_{\overline{c}}$ (ml./min) $\frac{Q-q}{\bar{c}t} \times 60$	$C_{\overline{i}} = RFR$ (ml./min) $\frac{Q}{\bar{i}t} \times 60$	% of free-flow GFR	(ml./8 min)	% of vol. in 8 min of free flow
A	10·5	9·17	28·8	2·90	14·96	49·7	6·21	2·36	8·2	23·2	10·0
B	9·9	6·67	25·8	4·29	9·55	24·0	3·00	0·50	2·0	12·1	5·6
C	10·4	8·00	24·2	2·69	12·21	34·4	4·30	1·95	8·0	18·6	10·8
D	14·5	10·00	34·3	3·47	11·50	46·8	5·85	2·38	6·9	25·1	9·2
E	14·5	8·30	20·3	2·42	12·24	34·0	4·25	1·13	5·6	17·8	9·2
Mean	—	—	26·7	—	—	—	4·72	1·66	6·14 S.D. ± 2·54	—	—

conditions (see Methods), showed that with a constant plasma creatinine concentration the average U/P ratios for the total occluded volume were 1·05–1·20 times the free-flow ratios. In the five experiments under consideration in Table 1 this would correspond to maximal U/P ratios ranging from 2·95 to 4·65. It thus appeared that of the total volume of filtrate of which the occluded fluid was the residue between one quarter and three fifths entered the kidney after the ureter was clamped.

Residual filtration in the presence of high ureter pressures

Between the sixth and eleventh minutes of ureteric obstruction residual filtration (RFR) averaged 5·9% (S.D. ± 1·41) of the free-flow glomerular filtration rate (GFR) in six experiments in which diuresis was produced by infusing 23% mannitol. In another six experiments the infusion fluid contained 10% mannitol and 15% glucose, and in this series the residual filtration rate averaged 11·4% (S.D. ± 1·65) of the free-flow GFR. The difference of 5·5% between the mean RFR values in the two types of experiment was very highly significant statistically ($P < 0·001$).

The rate of urine flow, free-flow GFR and creatinine U/P ratio, occluded volume, and arterial blood pressure did not differ systematically in the two series (Table 2). The final ureter pressure averaged 118 mm Hg when mannitol alone was infused, and 95 mm Hg when the mannitol-glucose mixture was given, a significant difference ($P < 0·05$). The final $A–U$ gradient, i.e. the pressure drop between femoral arterial blood and the fluid in the blocked ureter, averaged 37·5 mm Hg in the former, and 45·0 mm Hg in the latter series. The difference between these two values was not statistically significant, and the difference in ureteric pressure was in part a reflexion of the higher average arterial blood pressure in the experiments in which mannitol only was used.

The results revealed a genuine difference between the two types of experiment which was independent of whether the assumptions made in calculating RFR were correct or not. This difference showed that filtration in the occluded kidney was greatly influenced by the composition of the fluid trapped in the nephron, even if this fluid contained much osmotically active solute. The presence of a substance which could be actively reabsorbed from the tubular lumen clearly favoured the continuing entry of new filtrate in comparison with an essentially non-reabsorbable compound. It was interesting that the additional exogenous creatinine that appeared in the occluded fluid if glucose was infused was not confined to the 'proximal' urine fractions, but seemed to diffuse, or flow, into the distal segment also. The distribution of the total recovered material in the 'dead space' was remarkably similar whether or not glucose was used. This is shown by the open and filled circles of Fig. 3*b*.

In phlorrhizinized animals (170–200 mg/kg) the difference between experiments with and without glucose disappeared. The results could not be compared directly with the findings in normal animals because of the profound depression of free-flow GFR by phlorrhizin.

TABLE 2. Residual filtration between the sixth and eleventh minute of ureteric obstruction

		Free flow			Ureteric obstruction					
						RFR		Final pressures (mm Hg)		
No.	Body weight (kg)	Urine (ml.)	GFR (ml.)	*U/P* creatinine	Occluded volume (ml.)	(ml./min)	% of free-flow GFR	A.B.P.	Ureter	*A–U*
				I. 23% mannitol infused						
*N*2	13·8	8·7	22·85	2·64	12·40	1·55	6·8	154	116	38
*N*3	11·8	9·0	26·80	2·98	16·05	1·59	5·9	142	109	33
*N*4	17·0	7·5	24·15	3·26	9·14	1·05	4·3	168	113	55
*N*6	18·5	16·2	45·50	2·80	20·33	1·98	4·3	160	124	36
*N*8	14·3	11·5	38·18	3·32	19·70	2·53	6·6	154	117	37
*N*19	16·2	11·8	34·10	2·89	18·18	2·67	7·8	156	130	26
Mean	15·3	10·78	31·93	2·98	15·97	1·89	5·95	156	118	37·5
S.D.	±2·4	±3·14	±8·91	±0·26	±4·41	±0·62	±1·41	±8·5	±7·6	±9·6
				II. 10% mannitol and 15% glucose infused						
*G*1	15·0	10·2	31·21	3·06	15·57	4·09	13·1	160	87	73
*G*2	11·8	8·0	28·80	3·60	18·00	2·88	10·0	122	75	47
*G*3	15·7	11·5	33·35	2·90	14·98	3·99	12·0	154	118	36
*G*4	13·5	12·0	33·36	2·78	15·42	3·85	11·5	144	102	42
*G*5	12·1	12·2	31·48	2·58	15·76	4·07	12·9	136	92	44
*G*7	19·2	13·0	46·30	3·56	25·08	4·12	8·9	144	116	28
Mean	14·55	11·15	34·08	3·08	17·47	3·83	11·40	143	98	45
S.D.	±2·75	±1·80	±6·22	±0·42	±3·89	±0·47	±1·65	±13·4	±16·9	±15·3
				III. 23% mannitol infused. Hydrochlorothiazide, I.V.						
*C*9	15·5	9·33	30·32	3·25	20·43	3·43	11·3	138	89	49
*C*10	17·8	7·00	20·58	2·94	17·02	1·81	8·8	114	84	30
*C*18	12·2	16·30	33·90	2·08	20·78	3·27	9·6	134	107	27
Mean	15·2	10·9	28·25	2·79	19·41	2·84	9·9	129	93	35
				IV. 23% mannitol infused. Mercaptomerin sodium, I.V.						
*M*11	10·1	10·50	22·36	2·13	11·00	1·03	4·6	126	105	21
*M*13	16·3	10·50	32·23	3·07	19·43	2·84	8·8	150	123	27
*M*16	13·8	11·00	25·66	2·33	15·03	2·04	7·9	150	112	38
Mean	13·4	10·70	26·75	2·51	15·15	1·97	7·1	142	113	29

In a further six experiments drugs known to diminish tubular reabsorption of sodium were used in order to determine whether residual filtration in the occluded kidney was thereby reduced or abolished. In all of them increased excretion of water and sodium and increased urinary sodium concentration in conditions of free urine flow showed the drugs to have been effective. Filtration during occlusion was not measured in the same animals before giving the drugs because a high background of creatinine

and inulin persisting in plasma and urine after the first run impaired the accuracy with which small changes of concentration could then be assessed in the second of two consecutive runs.

In three experiments 10–20 mg hydrochlorothiazide (Esidrex, Ciba) was injected intravenously after full diuresis had developed during infusion of 23% mannitol; the ureter was blocked 10–20 min later. Contrary to expectation RFR exceeded in every instance the highest single value found in the six experiments without the drug. It averaged 9·9% of the free-flow filtration rate compared with 5·95%, but none of the remaining measured variables showed any clear differences (Table 2/III).

The chlorothiazides are known to exhibit antidiuretic properties in certain conditions (Crawford & Kennedy, 1959). As this might conceivably have accounted for enhanced reabsorption of the static fluid in the occluded nephrons, and might thus have promoted the entry of new filtrate, a further three experiments were carried out in which sodium reabsorption was depressed by mercaptomerin sodium (Thiomerin, Wyeth): 2 mg Hg/kg was administered by slow intravenous infusion in the course of 45 min, followed by the usual rapid infusion of 23% mannitol. The ureter was blocked 40–50 min after stopping the drug infusion. Again RFR was found to be somewhat greater than when no drug was given, averaging 7·1% of free-flow GFR, but in this case there was some overlap between the groups (Table 2/IV). In no instance did mercaptomerin depress the residual filtration to a value below those found when sodium reabsorption was not inhibited. Neither drug enhanced RFR as much as did glucose.

Occlusion of the ureter during minimal osmotic diuresis

On a number of occasions the ureter was blocked while urine was excreted at much lower rates than in the experiments described so far. The osmotic activity of the anaesthetic by itself was responsible for unilateral flow rates between 0·1 and 0·35 ml./min; they could be increased to 1·0–2·5 ml./min by cautious infusion of 5% mannitol. In this type of experiment ureteric obstruction was maintained for periods ranging from 18 to 120 min. During this time changes in filtration rate of the contralateral kidney sometimes occurred spontaneously, and sometimes as a result of the experimental procedure. It was therefore necessary to compare the performance of the obstructed and unobstructed kidneys while plasma creatinine concentration was maintained at a *constant* raised level. The mean plasma creatinine clearance of each kidney (as distinct from 'filtration' rate), while one of them was occluded, was expressed as a percentage of the average creatinine clearance in all free-flow control periods. The course of one such experiment is illustrated in Fig. 6. In this

and in most other low-flow experiments the ureter was obstructed twice; on the first occasion occlusion lasted three times as long as the interval which elapsed before ureter pressure reached a constant level; on the second for the length of this interval only.

Sections from a typical ureter pressure tracing are shown in Fig. 7. After a rapid rise to 20–30 mm Hg immediately after clamping, the

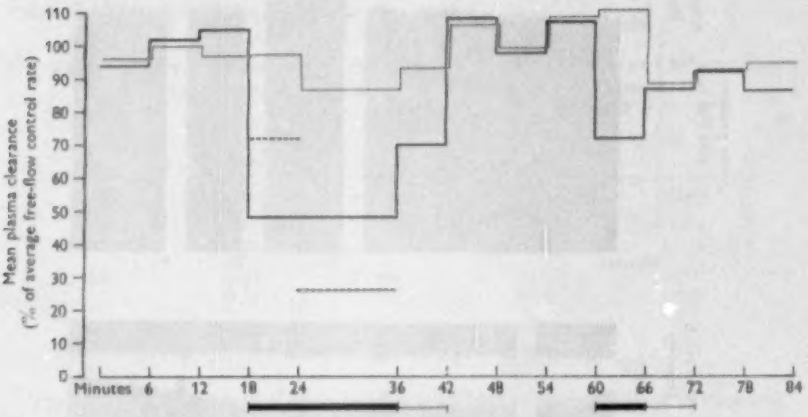

Fig. 6. Plasma creatinine clearance during slight osmotic diuresis in the freely draining and in the obstructed kidney. Thin line: right kidney, draining freely in all periods. Thick line: left kidney, whose ureter was blocked during two periods of unequal length, indicated by the heavy horizontal bars. Thin horizontal bars show periods of collection of fractional urine samples from left kidney. The mean plasma clearance for each period is calculated as a percentage of the average clearance during all free-flow periods for the same kidney. Plasma creatinine kept constant at a raised level by slow continuous infusion. Initial urine flows: left kidney, 2·17 ml./min; right kidney, 2·03 ml./min. Ureter pressure in the left kidney reached a constant level after 6 min obstruction. If on the first occasion mean plasma clearance during the initial 6 min was the same as on the second occasion (72 %), the calculated mean plasma clearance during the remaining 12 min of constant ureter pressure drops to 26 % of the control rate, as indicated by the broken lines.

pressure remained at or near this level for several minutes. It then climbed slowly and steadily and eventually reached a plateau. The time necessary for the level to become constant was greater the slower the rate of urine flow before occlusion. After this the only detectable further changes paralleled changes in blood pressure, i.e. the *A-U* pressure difference appeared to remain constant indefinitely. It was greater than when the kidney was occluded during maximal osmotic diuresis, varying from 115 to 80 mm Hg. If, after it had remained apparently unaltered for 30–90 min, a fast infusion of 20 % mannitol was begun, ureter pressure

would immediately start to rise again steeply, and the *A–U* gradient was diminished. This phenomenon was first briefly described by Winton (1935) and was seen during micropuncture studies on the unobstructed diuretic kidney by Gottschalk & Mylle (1957). It invariably occurred in the

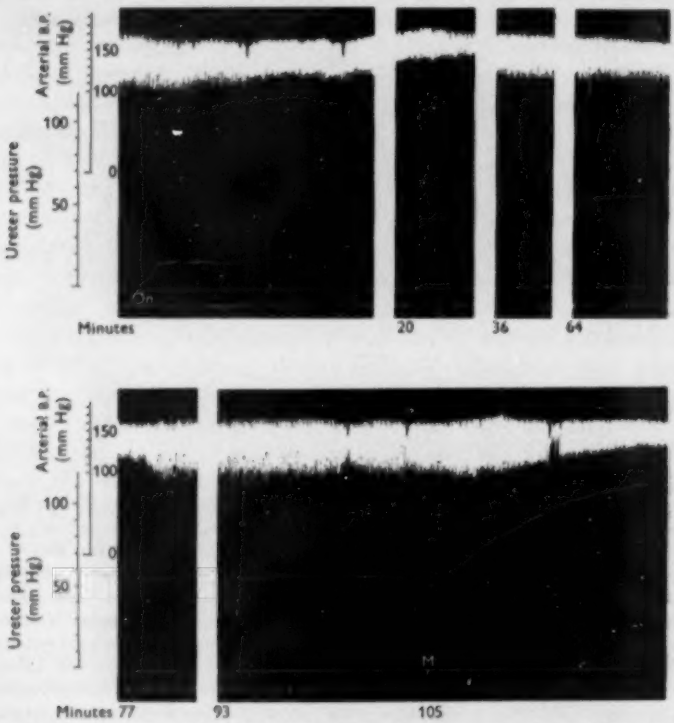

Fig. 7. Obstruction of the ureter during minimal osmotic diuresis. Sections from a continuous pressure record. Upper trace, blood pressure in femoral artery; lower trace, pressure in the blocked left ureter. Urine flow from left kidney before occlusion, 0·18 ml./min. No osmotic diuretic administered except chloralose-urethane mixture given as anaesthetic. 105 min after start of occlusion an infusion of 20% mannitol at the rate of 10 ml./min was begun (M). Ureter pressure began to rise almost at once and eventually levelled out again at a higher value.

present low-flow experiments after injection of the final filtration marker which was begun 90 sec and completed 30 sec before release of the obstruction; as already mentioned, it was seen also in vestigial form if the ureter was blocked during maximal osmotic diuresis.

Owing to the slow rates of urine flow a long time was required to collect, after occlusion, the volume of occluded fluid which was uncontaminated

by the final filtration marker. The latter tended to appear suddenly in relatively high but variable concentration, and in only three out of seven experiments was it possible to differentiate with some degree of confidence between 'occluded' volume and urine derived from filtrate formed after the release of the block. In these three experiments the mean plasma clearance during the long occlusion was approximately one half, and during the short occlusion approximately three quarters, of the average control value for the same kidney (Table 3).

TABLE 3. Mean plasma clearances in minimal osmotic diuresis. $C_{\overline{In}}$ expressed as percentage of average value for all free-flow control periods. Left ureter obstructed

No.	Free urine flow before occlusion (left kidney: ml./min)	Minutes of occlusion	$C_{\overline{In}}$ Left	$C_{\overline{In}}$ Right
L 3	2·17	18	47·7	92·4
	2·58	6	72·0	111·2
L 6	0·28	60	50·1	92·0
	0·58	20	69·4	96·8
L 7	0·20	45	63·1	97·2
	0·18	17	95·6	92·1

These results demonstrated that in a kidney whose outflow is blocked while the urine flow is low filtration may continue at a considerable rate, even at a time when there is no further obvious change in ureter pressure. This conclusion was implicit also in the renewed rise of ureter pressure following injection into the blood stream of non-reabsorbable material. The simultaneous diminution of the *A–U* gradient indicated that there was now osmotic retention in the nephron of fluid that could be continuously reabsorbed during the preceding phase, in which ureter pressure had remained steady at a lower level. But to exert this effect non-reabsorbable material which is not secreted must clearly be able to pass into the nephron through the glomerular membrane.

DISCUSSION

Ureter pressure and filtration in the occluded kidney

During osmotic diuresis the intratubular pressure in the distended nephrons is greatly elevated compared with the non-diuretic state, and is almost as high in the distal as in the proximal segments. At the same time the pressure in the renal pelvis is not increased, so that the pressure drop across the collecting duct system becomes much steeper. This was shown by Gottschalk & Mylle (1957) in direct micropuncture studies on the freely draining kidney of the rat. If the state of the dog's kidney in osmotic

diuresis resembles that of the rat the very rapid rise of ureter pressure seen immediately after occlusion probably represents the instantaneous equilibration of the low pelvic pressure with the existing high pressure in the proximal and distal tubules, and not, as is usually supposed, the filling up of the system with fluid that cannot escape. When creatinine was injected at the moment of occlusion the amount of exogenous material that found its way into the occluded nephrons was much greater than after later injection, but this does not necessarily imply that the volume contained in the system increased during the first minute or two. If before most of the normal reabsorptive processes came to a standstill the tubules continued for a brief time after ureteric obstruction to reabsorb, as during free flow, 50–60 % of the glomerular filtrate, a large quantity of fresh filtrate charged with injected creatinine could replace the fluid reabsorbed without any change in the total volume of fluid in the nephrons.

The subsequent gradual rise of ureter pressure, on the other hand, is almost certainly due to a slow increase of intratubular volume. It suggests steady accumulation of the non-reabsorbable residue of glomerular filtrate formed at this stage. Without knowledge of the actual glomerular capillary pressure and of the capacity of the system of nephrons it is not possible to tell from the ureter pressure curve and $A–U$ difference alone whether at this time filtration proceeds at a steadily diminishing or at a constant rate. If the rising intratubular pressure reduced the net filtration pressure across the glomerular membrane, or if it depressed the remaining reabsorptive processes which make room for new filtrate, filtration rate would be falling. But filtration could continue at a constant rate if fluid leaked out of the tubules (as discussed below), or if the pressure rise within the tubules was transmitted to surrounding blood vessels, and thence backward to the glomerular capillaries, so that the effective gradient across the membrane remained unaltered. Our findings are in favour of a constant filtration rate at this stage, since the mean plasma clearances of inulin and creatinine were of the same order of magnitude whether injections were made 2 min or 6 min after blocking the ureter.

Ureter pressure and $A–U$ difference eventually attain constant values; how soon, and at what absolute level of pressure depends on the osmotic activity of the filtrate before and during ureteric obstruction. The plateau was usually, but not invariably, reached at about the sixth minute of occlusion during maximal osmotic diuresis. However, the flattening of the pressure curve is not a valid sign that filtration has ceased. For not only do glomerular substances still find access to the tubules at this stage, but the pressure in the ureter can be made to rise further by suddenly raising their concentration in the circulating plasma. This is difficult to explain unless the osmotic activity of glomerular substances on entering

the tubules retains in the nephrons fluid which also entered, but was not so retained during the preceding 'steady' state.

The higher residual filtration rates encountered if glucose was substituted for part of the mannitol in the filtrate were clear evidence that continuing active reabsorption is an important factor determining filtration rate in these conditions. Part of the removal of fluid from the nephrons which permits continued filtration in the occluded kidney appeared however to be due to a physical mechanism, such as the difference in protein concentration between lumen and post-glomerular blood, or to 'distal' water reabsorption. The experiments which showed that inhibitors of sodium reabsorption did not reduce the rate of residual, i.e. 'replacement' filtration, though not conclusive, strongly suggested this.

In all conditions explored some fraction of the newly entering filtrate would normally be left behind in the tubules as non-reabsorbable residue. Thus if ureter pressure can become constant while filtration continues, a state of equilibrium must have been reached, but in what manner is far from clear.

If the tubules increased their capacity at the expense of the blood content of adjoining capillaries and venules the process would be rapidly self-limiting. It may be argued that the rate of deposition of non-reabsorbable material from the filtrate has become so slow that the associated rise of ureter pressure escapes detection in short periods of observation. But it was not possible to detect it even when the occlusion was continued for 50–60 min after an apparently constant pressure was reached during mannitol infusion, and for 120 min when no infusion was given. This failure was not conclusive proof against the hypothesis, since the rise may not have manifested itself because the animals' condition may have deteriorated during these long experiments. In an intact animal in the course of hours or days the pressure might well rise further.

Another possibility is that ureter pressure became constant because the 'non-reabsorbable residue' becomes reabsorbable after obstruction of the ureter. This was suggested and discussed by Winton (1935), who thought that there might be a leak from the distal segment. The distribution of the filtration marker after late injection in our experiments makes it more probable that leakage, if it occurs, is from the proximal tubule. Nor can it be a simple escape of fluid through holes in the tubular wall. The cells of the proximal tubule become thin and flattened when there is a high hydrostatic pressure in the lumen (Brodie, 1914; Gottschalk & Mylle, 1957). This, or stagnant anoxia in the congested kidney, might conceivably destroy their power to prevent selectively the outward passage of certain molecules, so that all the filtrate formed could then be returned to the blood stream by active transport or protein osmotic pressure. However, certain difficulties arise in this context. The postulated loss of selectivity

has to be reconciled with the fact that the tubular wall appears to remain an effective semipermeable membrane with respect to the osmotic activity of the fluid entering the nephrons, and of the protein in the post-glomerular blood. Also, as Winton has pointed out, if all substances become reabsorbable, including inulin and creatinine, filtration rate will be underestimated. The validity of the inulin and creatinine clearance as a measure of filtration at low urine flows is still a matter for debate (Bálint & Forgács, 1960). The depression of these clearances during osmotic diuresis in the freely draining kidney (Mudge, Foulks & Gilman, 1949; Dempster, Eggleton & Shuster, 1956), though open to several interpretations, also indicates that the possible reabsorption of inulin and creatinine in the distended kidney must be seriously considered. However, there is as yet no definite proof that it occurs.

The validity of quantitative estimates of filtration rate in the obstructed kidney

It will be evident from the foregoing discussion that the assumptions made in the calculation of actual filtration rates were no more than working hypotheses, based chiefly on the mean plasma clearances of the injected substances. For creatinine administered at the instant of occlusion C_{in} over 8 min averaged 18 % of the free-flow GFR; and 6·1 % over the remaining 6 min of the same experiments for inulin injected 2 min later. When the creatinine injection was made 6 min after the start of occlusion in another group of animals under comparable conditions, C_{in} over 5 min averaged 5·9 %. From this it was inferred that the rate of entry of new filtrate into the blocked kidney probably altered very little between the second and the eleventh minutes, and even if it did diminish somewhat toward the end of the period no great error would be introduced by regarding the rate as constant. In the first 2 min, taken by themselves, C_{in} must have been much greater than 18 %, but the mean plasma concentration of the marker was high at that time. Therefore the true filtration rate must have been decreasing rapidly at first, and an exponential decline was assumed as most probably correct for the initial phase. It was realized that this would represent the actual course of the change of filtration rate only approximately; but it was considered that any divergence there might be would not substantially affect the values for total entry of filtrate during occlusion which it was desired to estimate.

If in the obstructed kidney creatinine and inulin were not dealt with in the normal way this would invalidate our estimates in a much more unpredictable manner than errors arising from the assumptions on which the calculations were based. That filtration rate might have been *under*-estimated has already been discussed. It might also have been

over-estimated if creatinine were secreted or diffused into the occluded tubules. Despite the initially high plasma concentrations this probably did not occur, as it made no difference whether in the same type of experiment creatinine or inulin was employed; it is not likely that both were secreted. An erroneously high value for filtration would also have been obtained if, owing to congestion, the injected material had lain stagnant in the glomerular capillaries in high concentration, to be swept into the filtrate after release of the ureter at a time when the systemic arterial concentration had fallen to a much lower value. That this was not the true explanation for the higher concentration of the marker in the 'occluded' volume was shown by the fact that the amounts of creatinine which entered it differed greatly in experiments with or without glucose, which were alike in every other respect.

Implications for stop-flow analysis

For the practice of stop-flow analysis it is not without interest that glomerular filtration in the occluded kidney does not stop, even when ureter pressure reaches a steady level, an event which does not take place before the fifth or sixth minute of occlusion. Omachi & Macey (1959), in a brief report not seen by us before our work was completed, have described the presence in occluded fluid of various filtration markers injected successively during a stop-flow experiment. Their findings agree with ours, but in their deductions they do not appear to have taken into account that the *concentration* of the recovered marker is no direct guide to the volume of filtrate in which it was contained. It is apparent from our results that 'flow' during ureteric obstruction is restricted in the main to the upper portions of the nephrons. As during free urine flow, the volume of fluid reabsorbed from the distal segment and replaced from above must be very small in comparison with filtration. The fluid in the distal 30–40 % of the occluded fluid column, which usually includes the region of lowest sodium concentration, appears to be only very slightly contaminated by filtrate that has entered the proximal tubules after the start of occlusion; virtually no filtrate formed after the second minute reaches it. Thus in the distal segment reabsorption and secretion do indeed act on an almost stationary pool.

More proximal sites receive during occlusion progressively increasing amounts of new filtrate. Samples coming from these regions are therefore not in their entirety contemporaneous with the distal samples, nor within their own sequence. The arrival of new filtrate will make secretory processes appear exaggerated and will tend to conceal reabsorption. This can in part be amended by using the ratio $U/P_{(x)}:U/P_{(\text{creatinine})}$ when following the fate of substance x. But this correction does not eliminate inequalities between portions of the contents which are due to differences in time spent

in the tubules. That the interpretation of distal concentration patterns is more reliable and straightforward has, of course, been inferred from indirect evidence and other arguments by several users of the method.

From a study of the creatinine concentration of proximal samples Malvin, Wilde, Vander & Sullivan (1958) obtained the impression that replacement of occluded fluid by fresh filtrate was enhanced by substances like glucose which can be actively reabsorbed. Our findings have confirmed that this is in fact the case. It follows that if the composition of the solution infused is altered between two consecutive experiments performed on the same animal the two runs may not be strictly comparable, because of differences in the amount of filtrate formed during occlusion which will penetrate to a given distal or proximal site.

If the outflow is obstructed when no or only a minimal quantity of an osmotic diuretic is circulating filtration in the occluded kidney continues at such a high rate that stop-flow patterns obtained in these circumstances would appear to be meaningless. By imposing on the ureter from the start of occlusion a pressure close to or higher than arterial pressure, as was recently done by Jaenike & Berliner (1960), this difficulty can probably be overcome.

SUMMARY

1. By a modification of stop-flow analysis the amount and distribution of exogenous creatinine and inulin in the volume of fluid occluded in the dog's kidney were determined. These substances were injected into the circulation at various times after obstruction of the ureteric outflow during maximal osmotic diuresis. The changes in their plasma concentration were followed, and the pressure in the ureter above the obstruction was continuously recorded.

2. Ureter pressure was found to rise rapidly at first and then slowly and steadily. It did not attain a constant level until 5 min after obstruction, or later.

3. The levelling of the pressure curves did not coincide with cessation of glomerular filtration, which was found to continue throughout periods of occlusion lasting up to 11 min.

4. On the basis of specified assumptions the total volume of filtrate entering the obstructed kidney during 8 min of occlusion was estimated to average 9% of the volume of filtrate that would have been formed in 8 min of free urine flow.

5. No systematic difference in the plasma clearance of filtration markers was detected whether they were injected 2 or 6 min after blocking the ureter. It was inferred that after the second minute of occlusion filtration continued at an approximately constant rate, averaging 5·9% of the free-flow rate if 23% mannitol was infused. The rate averaged 11·4% of the

free-flow value if two thirds of the mannitol in the infusion was replaced by glucose.

6. From 96 to 100% of the occluded fluid was found to contain material that had crossed the glomerular membrane after the ureter was obstructed. The distal 30–40% of the occluded fluid column received a small fraction of the filtrate formed during the first 2 min, and practically none of that formed after the sixth minute. Considerable quantities of new filtrate continued to flow into the proximal third of the 'dead space'.

7. If the ureter was blocked for 18–120 min when no or little osmotic diuretic was circulating the occluded fluid contained up to 75% of the quantity of filtration marker excreted in the same time by the freely draining kidney. Ureter pressure became constant later, and at a lower absolute value than in maximal osmotic diuresis, but this again did not indicate cessation of filtration.

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REFERENCES

BÁLINT, P. & FORGÁCS, I. (1960). Zur Bewertung der Inulinclearance. *Pflüg. Arch. ges. Physiol.* **271**, 23–34.

BRODIE, T. G. (1914). A new conception of glomerular function. *Proc. Roy. Soc.* B, **87**, 571–592.

CRAWFORD, J. D. & KENNEDY, G. C. (1959). Chlorothiazid in diabetes insipidus. *Nature, Lond.*, **183**, 891–892.

DEMPSTER, W. J., EGGLETON, M. G. & SHUSTER, S. (1956). The effect of hypertonic infusions on glomerular filtration rate and glucose reabsorption in the kidney of the dog. *J. Physiol.* **132**, 213–224.

FORLDI, M., PAPP, N. & KOLTAY, E. (1958). Nierenfunction in der Hydronephrose. *Folia angiol.* **5**, 3–14.

GILMAN, A., PHILIPS, F. S. & KOELLE, E. S. (1946). The renal clearance of thiosulfate with observations on its volume distribution. *Amer. J. Physiol.* **146**, 348–357.

GOTTSCHALK, C. W. & MYLLE, M. (1957). Micropuncture study of pressures in proximal and distal tubules and peritubular capillaries of the rat kidney during osmotic diuresis. *Amer. J. Physiol.* **189**, 323–328.

JAENIKE, J. R. & BERLINER, R. W. (1960). A study of distal renal tubular functions by a modified stop-flow technique. *J. clin. Invest.* **39**, 481–490.

JANCSÓ, N. (1955). *Speicherung*, pp. 242–266. Budapest: Akademiai Kiado.

MALVIN, R. L., WILDE, W. S. & SULLIVAN, L. P. (1958). Localization of nephron transport by stop flow analysis. *Amer. J. Physiol.* **194**, 135–142.

MALVIN, R. L., WILDE, W. S., VANDER, A. J. & SULLIVAN, L. P. (1958). Localization and characterisation of sodium transport along the renal tubule. *Amer. J. Physiol.* **195**, 549–557.

MUDGE, G. H., FOULKS, J. & GILMAN, A. (1949). Effect of urea diuresis on renal excretion of electrolytes. *Amer. J. Physiol.* **158**, 218–230.

OMACHI, A. & MACEY, R. I. (1959). Intratubular fluid movement in dog kidney during stop-flow. *Proc. Soc. exp. Biol., N.Y.*, **101**, 386–388.

SCHREINER, G. E. (1950). Determination of inulin by means of resorcinol. *Proc. Soc. exp. Biol., N.Y.*, **74**, 117–120.

WINTON, F. R. (1935). The relation between diuresis and maximum ureter pressure, and its bearing on current methods of estimating the rate of glomerular filtration. *J. Physiol.* **83**, 38–39*P*.

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ACUTE INSULIN DEFICIENCY PROVOKED IN THE DOG, PIG AND SHEEP BY SINGLE INJECTIONS OF ANTI-INSULIN SERUM

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It has been reported that the intravenous or intraperitoneal injection of guinea-pig anti-insulin serum provokes hyperglycaemia in the mouse (Moloney & Coval, 1955; Moloney & Goldsmith, 1957), and in the rat, rabbit and cat (Armin, Grant & Wright, 1960*a*, *b*). It is now shown that such serum has a similar effect in dogs, a pig and a lamb and that in the pig and the lamb this hyperglycaemia coincides with the disappearance of biologically active insulin from the blood.

METHODS

Sera. Guinea-pig anti-insulin serum was obtained from animals treated with recrystallized bovine insulin (Robinson & Wright, 1961) and assayed for insulin-neutralizing potency in conscious fed rats (Armin *et al.* 1960*a*). Control serum was obtained from guinea-pigs which had received injections of an emulsion containing no added insulin, whilst normal serum came from untreated normal animals.

Animals. Dogs: three mongrel dogs (No. 1, male, 17 kg; No. 2, male, 13 kg; No. 3 female, 17 kg) were anaesthetized with thiopentone sodium (Pentothal, Abbott Laboratories; 25 mg = 0·5 ml./kg).

Pig: a Large White pig (male; 26 kg) with pentobarbitone sodium (Veterinary Nembutal, Abbott Laboratories; 30 mg = 0·5 ml./kg) anaesthesia being maintained by smaller doses injected intravenously as required. Local procaine (2 g/100 ml.) anaesthesia was used to introduce polythene catheters (internal diameter, 0·023 mm., size PE10, Clay Adam Co. Inc.) into a vein and an artery; the femoral vessels were used in the dogs and smaller superficial vessels in the hind limb of the pig.

Lamb: a Hampshire lamb (female, 24 kg) had a similar catheter inserted through a needle into the external jugular vein; this animal received no general anaesthetic and was restrained in a specially constructed stock. The catheters were connected by way of Perspex three-way taps (Armin & Grant, 1953) to an infusion pump (Armin *et al.* 1960*b*) and were kept patent by slow infusions of NaCl solution (0·9 g/100 ml.). Fluids were injected and blood samples drawn through these taps, which also permitted measurement of mean arterial pressure with the mercury manometer.

Sugar estimations were carried out on blood samples (0·2 ml.) by a modification (Wright, 1957) of the method described by King (1951) and on samples of plasma and all incubation

media by the iodometric method of Somogyi (1952) after precipitation of the proteins with $Ba(OH)_2$ and $ZnSO_4$ (Somogyi, 1945). Both methods were used to determine the sugar content of some of the blood samples and similar results were obtained.

Plasma-insulin activity. The experimental procedure used to determine the glucose consumption rates of rat hemi-diaphragms was similar to those described by Vallance-Owen & Hurlock (1954) and Wright (1957); a more detailed account will be published elsewhere. Albino rats of a Wistar strain (125–200 g) were deprived of food but not of water for 24 hr and decapitated. The hemi-diaphragms were quickly removed and soaked for 10 min at room temperature (20–25° C) in a bicarbonate-buffered medium (Gey & Gey, 1936) containing glucose 0·25 g/100 ml. Each hemi-diaphragm was blotted and placed in the incubation medium (1 ml.) in a flask (gassed with a mixture of 95 % O_2 and 5 % CO_2) and then incubated at 38° C for 90 min. The glucose content of the incubation medium was determined before and after incubation and the hemi-diaphragm weighed in the wet state after incubation. Glucose consumption rates are expressed as milligrams glucose removed from the medium per gram wet weight of tissue per hour of incubation.

In each experiment 24 rats were used, 6 hemi-diaphragms from different rats being incubated separately in each medium. The basal medium was that described by Gey & Gey (1936) saturated at room temperature with gelatin (Davis Gelatine Co. Ltd). All media used for incubation contained glucose in a concentration of approximately 250 mg/100 ml.

Hemi-diaphragms incubated in the basal medium consumed glucose at the 'basal rate'. When insulin was added (0·01–1·00 m-u./ml.) more glucose was consumed, the increment being described as the 'insulin effect'; the stimulant effects produced by insulin in these concentrations were almost the same as those reported by Vallance-Owen & Hurlock (1954) and by Wright (1957, 1960*a*). Insulin-like activity in plasma and plasma extracts was indicated when hemi-diaphragms incubated in their presence consumed more glucose than others incubated in the same experiment in the basal medium. For incubation plasma was used after dilution (1:4) in the basal medium. Alternatively the plasma (2 ml.) was extracted by the method of Baird & Bornstein (1957) and the extract (lamb, 60–75 mg; pig, 37 mg) dissolved in the basal medium (8 ml.).

RESULTS

Dogs. Two animals (Fig. 1, Nos. 1 and 3) received intravenous injections of normal guinea-pig serum (1 ml./kg) followed later by a similar volume of anti-insulin serum (Batches 34943 and 479566) sufficient to neutralize 1·5 and 1·8 u. insulin/kg body wt. The third animal (Fig. 1, No. 2) received only anti-insulin serum (Batch 34943) in the same dosage. Figure 1 shows that neither of the dogs which received normal serum showed any change in blood-sugar concentration during the ensuing hour. In dog No. 1, however, the blood pressure fell from 170 to 83 mm Hg during the first 10 min and then rose to its original level in the next 30 min. No change in blood pressure was observed in dog No. 3. Following injection of anti-insulin serum the blood-sugar concentration rose in two dogs (Fig. 1, Nos. 2 and 3) but was unaffected in the third (Fig. 1, No. 1); the blood pressures did not change. When the experiments were ended approximately 1 and 4 hr after injection of the anti-insulin serum, the two responding dogs (Nos. 2 and 3) were still hyperglycaemic.

Pig. Figure 2 shows that control guinea-pig serum (Batch 64; 18 ml.)

provoked no significant change in blood-sugar concentration. A steady increase in mean arterial pressure from 80 to 115 mm Hg occurred in the first 40 min. Anti-insulin serum (Batch 479566; 26 ml.), sufficient to neutralize about 1·5 u. insulin/kg body wt. and injected 63 min later, produced a progressive increase in blood-sugar concentration from 80 to

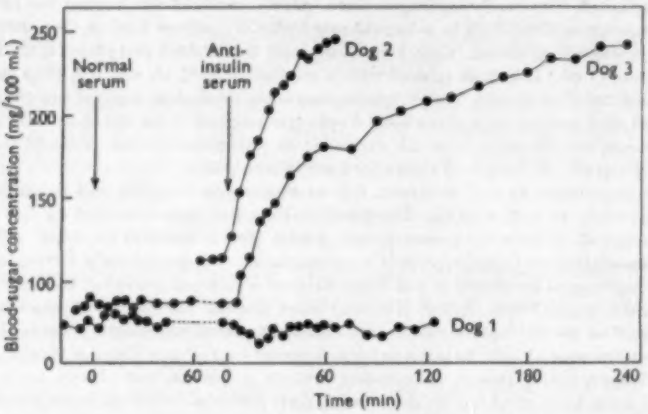

Fig. 1. Blood-sugar concentrations in anaesthetized (Nembutal) mongrel dogs after intravenous injection with normal guinea-pig serum and 46 min (Dog No. 1) and 63 min (Dog No. 3) later, with anti-insulin serum sufficient to neutralize about 1·5–1·8 u. insulin/kg body wt.; Dog No. 2 received only the anti-insulin serum.

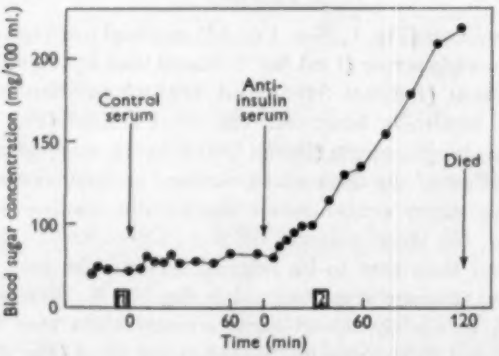

Fig. 2. Blood-sugar concentrations in an anaesthetized (Nembutal) Large White pig injected intravenously with control serum and 63 min later, with anti-insulin serum sufficient to neutralize 1·5 u. insulin/kg body wt. Blood samples (50 ml.) were drawn for plasma-insulin assay less than 10 min before control serum injection (No. 1) and 31 min after the injection of the anti-insulin serum (No. 2).

218 mg/100 ml. in 2 hr; mean arterial pressure remained constant (110–120 mm Hg) except after the collection of blood sample No. 2 when it fell temporarily. The animal died suddenly 2 hr after the injection of the anti-insulin serum and several minutes after an intravenous dose of pentobarbitone; the cause of death appeared to be acute respiratory failure.

Diluted plasma obtained from the heparinized blood sample (No. 1, 50 ml.), drawn immediately before the injection of control serum, produced a small but significant insulin effect when assayed by the rat-diaphragm method. The extract obtained from the same sample was slightly more active. These effects, summarized in Table 1, contrast with those produced by the plasma and the extract obtained from the blood sample (No. 2, 50 ml.) drawn 30 min after the injection of the anti-insulin serum. The diluted plasma in this case was inactive, whereas the extract was about three times more active than the extract from the first sample.

TABLE 1. Glucose consumption rates* of rat hemi-diaphragms incubated in the presence of diluted plasma and plasma extracts from a pig before and 30 min after injection with anti-insulin serum

Material assayed	Glucose concn. (mg./100 ml.)	Incubation medium: Basal (*a*)	Incubation medium: Basal + assay material (*b*)	Insulin effect (*b* − *a*)	Significance of insulin effect
Plasma No. 1	104	2·20 (0·06)	2·74 (0·21)	0·54 (0·22)	$0·02 < P < 0·05$
Plasma No. 2	141	2·20 (0·06)	2·41 (0·08)	0·21 (0·10)	$0·05 < P < 0·10$
Extract No. 1	—	2·66 (0·13)	3·53 (0·16)	0·87 (0·21)	$P < 0·01$
Extract No. 2	—	2·66 (0·13)	5·33 (0·24)	2·67 (0·27)	$P < 0·001$

* Glucose consumption rates stated as mg glucose/g wet wt. tissue/hr; S.E. of means in parentheses.

Lamb. When normal guinea-pig serum (1 ml./kg) was injected into the lamb no change in blood-sugar concentration was produced. Anti-insulin serum (Batch 479566; 24 ml.), sufficient to neutralize about 1·5 u. insulin/kg body wt. and injected 46 min later, raised the blood-sugar level from 60 to 220 mg/100 ml. in 4 hr (Fig. 3). It remained elevated for at least 6 hr, but returned to normal limits within 24 hr and thereafter remained normal. The insulin effects produced by diluted plasma and plasma extracts obtained from blood samples drawn at various times during this experiment are given in Table 2 and Fig. 3. Detectable activity was found in diluted plasma obtained before (No. 1) and more than 6 hr after (Nos. 7–13) anti-insulin serum injection; those obtained less than 6 hr after injection of this serum (Nos. 3 and 6) were inactive. The insulin effect of diluted plasma No. 7 was significantly greater than that of plasma No. 1 ($P < 0·001$) and those of samples (Nos. 8–13) drawn during the succeeding month ($P < 0·01$). The insulin effects of plasma extracts Nos. 2–11 were significantly greater than that of plasma extract No. 1 ($P < 0·001$) and

those of extracts Nos. 12 and 13. The extract of plasma obtained from this lamb's twin at the same time as plasma extract No. 13 had an insulin effect of $2{\cdot}18 \pm 0{\cdot}30$ mg glucose/g/hr; the effects produced by these two extracts are not, therefore, significantly different.

Throughout the time this animal was restrained in the stocks (about 6 hr) it showed no signs of distress and was not unduly restless; it ate food when this was offered and lay down on two occasions. Once released to pasture its behaviour was not detectably different from that of its twin.

TABLE 2. Insulin effects (assayed by the rat-diaphragm method) produced by diluted plasma and plasma extracts from a lamb before and up to 29 days after injection with anti-insulin serum

Sample no.	Time of collection†		Glucose concn. (mg./100 ml.)	Insulin effects*			
	hr	days		Diluted plasma	Significance	Plasma extract	Significance
1	Control		86	0·52 (0·21)	$0{\cdot}02 < P < 0{\cdot}05$	0·87 (0·31)	$0{\cdot}02 < P < 0{\cdot}05$
						0·88 (0·15)	
2	1½		164	—	—	3·27 (0·38)	
3	2		214	0·02 (0·16)	Nil	3·13 (0·29)	
4	3		268	—	—	3·26 (0·30)	
5	4½		296	—	—	3·74 (0·21)	
6	4½		297	0·03 (0·26)	Nil	3·27 (0·28)	
						3·45 (0·21)	
7	6		286	2·35 (0·23)	$P < 0{\cdot}001$	3·96 (0·19)	$P < 0{\cdot}001$
8		1	88	0·44 (0·26)	$0{\cdot}10 < P < 0{\cdot}20$	3·65 (0·22)	
9		2	78	0·48 (0·21)	$0{\cdot}05 < P < 0{\cdot}10$	3·60 (0·27)	
10		6	100	1·15 (0·21)	$P < 0{\cdot}001$	3·48 (0·19)	
						2·97 (0·22)	
11		10	85	1·28 (0·24)	$P < 0{\cdot}001$	3·20 (0·35)	
12		15	84	0·31 (0·19)	$0{\cdot}10 < P < 0{\cdot}20$	2·31 (0·33)	
13		29	89	0·33 (0·16)	$0{\cdot}05 < P < 0{\cdot}10$	2·19 (0·30)	

* Insulin effects stated as mg glucose/g wet wt. tissue/hr; S.E. of mean in parentheses.

† Period stated is the time elapsing from the time of injection of anti-insulin serum to the time of blood collection. The control sample was collected 16 min before injection of the normal serum.

DISCUSSION

The effects produced by guinea-pig anti-insulin serum in the dog, pig and sheep are similar in many respects to those provoked in the rabbit, rat and cat (Armin *et al.* 1960*a*), but there are certain differences. Thus, only two of the three injected dogs (Fig. 1, Nos. 2 and 3) became hyperglycaemic. The dog which did not respond (No. 1) had received no food for at least 24 hr before injection. In the rat fasting is known to reduce the initial rate of increase in blood-sugar concentration from 3·16 to 1·78 mg/100 ml./min. The other two dogs had received liberal diets for at least 3 days before injection. In addition, these dogs were anaesthetized with barbiturate which, in the case of fasting but not of fed rats, reduced the rate of rise still further to 0·25 mg/100 ml./min. Thus, fasting superimposed upon barbiturate anaesthesia may be responsible for the failure

of this dog to respond to anti-insulin serum. The reason for this is not known, but it should be remembered before any conclusions are drawn from similar experiments in which injected animals fail to respond. The pig and lamb were both well nourished and fed up to the time of the experiment. A second difference is that whereas the blood sugar concentrations increased at similar initial rates (mg/100 ml./min) in injected conscious rats (3·16) and rabbits (3·04) and in the anaesthetized cat (4·0), slower rates were observed in the anaesthetized dogs (2·15 and 1·50) and pig (1·16) and

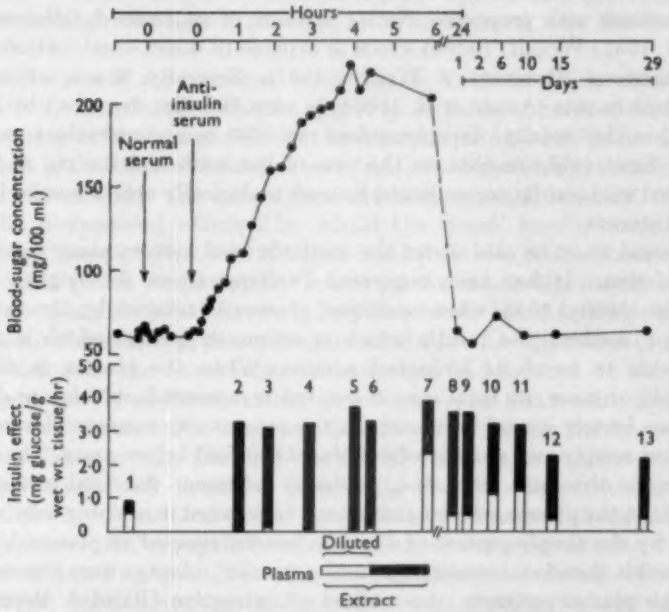

Fig. 3. Blood-sugar concentrations and insulin effects of diluted plasma and plasma extracts from a conscious Hampshire lamb injected with normal serum, and 46 min later with anti-insulin serum sufficient to neutralize 1·5 u. insulin/kg body wt. The numbers of the plasma samples (see Table 2) are shown above the insulin effects produced by them after dilution or extraction.

the slowest was found in the conscious lamb (0·67). Thirdly, the hyperglycaemia provoked by comparable doses of serum injected into these three animals was more prolonged than that in the rat. A dose sufficient to neutralize about 1·5 un. insulin/kg body wt. produces hyperglycaemia in the rat lasting about 55 min (Robinson & Wright, 1961), yet the larger animals were still markedly hyperglycaemic after 1–6 hr. The differences in response in these various animals may be due to differences in their

metabolic processes, their varying normal rates of insulin secretion or different degrees of endogenous insulin binding by the injected anti-serum; further investigations are required.

The metabolic changes produced in mice (Moloney & Coval, 1955) and in rats (Armin *et al.* 1960*b*) by the parenteral administration of guinea-pig anti-insulin serum in sufficient quantity are thought to be due to acute insulin deficiency produced by neutralization and hence inactivation of endogenously secreted insulin. Two pieces of indirect evidence support this hypothesis. First, it is known that such anti-insulin serum contains a substance with properties similar to those of an antibody (Moloney & Coval, 1955; Wright, 1960*b*) which is capable of direct combination with the hormone (Robinson & Wright, 1961). Secondly, the syndrome it produces in rats (Armin *et al.* 1960*b*) is very like that described by Scow (1957) in the 'totally' depancreatized rat. Our new observations provide more direct evidence that, in the case of the lamb and the pig at least, injected anti-insulin serum neutralizes all biologically active insulin in the blood stream.

A word must be said about the methods used here to assay insulin in the plasma. It has been suggested (Vallance-Owen & Wright, 1960; Wright, 1960*a*) that, when undiluted plasma is assayed by the rat-diaphragm method, the insulin which is estimated is that which is freely available to exert its biological actions. When the plasma is diluted fourfold or more, its total insulin content is measured; dilution probably releases loosely bound hormone. In the present experiments plasma was in short supply and was therefore diluted fourfold before assay. The assay values in this case, therefore, probably represent the total amount of insulin in the plasma which exists or can be released in a biologically active form by the simple process of dilution; insulin assayed in plasma by this method is therefore termed here 'active insulin'. Assays were also carried out on plasma extracts, the method of extraction (Baird & Bornstein, 1957) having been designed to extract any hormone in the plasma which may exist in a firmly bound and inactive form. Assays carried out on such extracts must therefore give a measure of the 'total insulin' in the plasma whether it exists in an 'active' or a bound inactive form. The method of insulin assay itself is known to be sensitive but is not considered to be very precise (Randle, 1956; Vallance-Owen & Wright, 1960). Undue emphasis should not be placed, therefore, upon the estimates of plasma-insulin concentration quoted below and calculated from the experimental observations in Tables 1 and 2. It could also be argued that this method of assay is not specific for insulin, but since some of the extracts were extremely active it was possible to show that most of this activity was probably due to the hormone. One of us (Wright, 1959) showed that

guinea-pig anti-insulin serum will inhibit the stimulant effect of insulin upon glucose consumption by the isolated rat-diaphragm. One extract of lamb serum (Table 2, Extract No. 10) which had a strikingly high stimulant effect (insulin effect, 2·97 ± 0·22 mg glucose/g/hr) was incubated with anti-insulin serum (Batch 34943; 2% v/v). Under these conditions the glucose consumption rate (3·05 ± 0·28 mg glucose/g/hr) was not significantly different from that of hemi-diaphragms incubated in the basal medium during the same experiment (2·78 ± 0·10 mg glucose/g/hr). This evidence, taken alone, is not conclusive proof but it is strong presumptive evidence that the active material in these extracts was the hormone itself.

In the case of the conscious lamb (Fig. 3; Table 2) which was studied more fully than the anaesthetized pig (Fig. 2; Table 1), most of the insulin circulating in the plasma before the injection of anti-insulin serum was present in the 'active' form (0·1 m-u./ml.); the 'total' insulin content was then 0·14 m-u./ml. Once the serum had been injected, however, 'active' insulin disappeared within 2 hr, whilst the 'total' insulin content rose to a significantly ($P < 0·001$) higher level (0·88 m-u./ml.). This suggests that the injected serum rapidly bound all the circulating insulin and all that the pancreas could secrete in response to the initial hyperglycaemia. No 'active' insulin was detected again until 6 hr after the serum injection, by which time the blood-sugar concentration had reached its maximum and presumably all the circulating guinea-pig serum had become saturated with bound insulin. The reappearance of 'active' insulin in high concentration (0·4 m-u./ml.) preceded the fall in blood-sugar concentration to normal levels and thereafter essentially normal concentrations were found (0·05–0·20 m-u./ml.). The 'total' insulin content of the plasma did not, however, fall as rapidly; concentrations of the order of 0·7–2·7 m-u./ml. were found up to 10 days after the injection of the serum. After this the 'total' insulin contents (0·4 m-u./ml.) were comparable with that of an extract of plasma taken at the same times from this lamb's twin, which had received no anti-insulin serum. This finding strongly suggests that the active component of the anti-insulin serum, probably an antibody of insulin, remains in an active form in the blood stream for several days. Once saturated with endogenously secreted insulin, however, it does not appear to interfere with normal insulin action. These conclusions also appear to be applicable, in part at least, in the case of the anaesthetized pig.

A lack of species-specificity in the inhibitory effect of guinea-pig anti-insulin serum has been demonstrated *in vitro* (Wright, 1959; Robinson & Wright, 1961) and *in vivo* (Moloney & Coval, 1955; Moloney & Goldsmith, 1957; Robinson & Wright, 1961). Berson & Yalow (1959), however, consider that the methods used in these experiments are too crude to

display fine differences in specificity. By their chromatographic-electrophoresis technique they find that human anti-insulin serum binds bovine and sheep insulin slightly more strongly than it does pig and horse insulin. Further, in developing an immunological method of plasma-insulin assay, they show that guinea-pig anti-insulin serum binds bovine insulin four times as strongly as it does human insulin (Yalow & Berson, 1960). The effects of active serum upon the actions of insulin extracted from the pancreas were examined in all the instances mentioned above, and it is known that the chemical compositions of the insulins extracted from the pancreas of the ox, horse, pig and sheep do differ slightly (Harris, Sanger & Naughton, 1956). It is not surprising, therefore, to find that at best only slight differences in immunological properties have thus far been reported in these instances. More important in the present context is the suggestion of Moloney & Coval (1955) that 'native' or endogenously secreted insulin may differ in its composition from 'altered' insulin extracted from the pancreas by means of acid-alcohol. The present results suggest that this is not so, for serum obtained from an animal treated with 'altered' bovine insulin not only inhibits the effects of 'altered' pig and sheep insulin *in vivo* (Moloney & Coval, 1955) and *in vitro* (Wright, 1959) but also neutralizes the endogenous insulin which these two animals secrete. Thus the 'native' insulins which they secrete are probably very similar in chemical composition to one another, to 'altered' bovine insulin, and to 'altered' sheep and pig insulins. The evidence obtained in previous experiments (Armin *et al.* 1960*a*) now makes it more likely that the endogenous insulins secreted by the rat, rabbit and cat will also prove similar in structure to one another and to endogenous dog, pig and sheep insulins. It remains to be seen whether human insulin, endogenous or extracted, differs more markedly in structure as the results of Yalow & Berson (1960) suggest.

SUMMARY

1. Guinea-pig anti-insulin serum injected intravenously into two dogs, a pig and a lamb provoked hyperglycaemia; one dog did not respond, probably owing to the combined effects of fasting and barbiturate anaesthesia.

2. Insulin-like activity in diluted plasma and plasma extracts obtained from the lamb and the pig was assayed on the isolated rat diaphragm.

3. It is concluded that, in the case of the lamb at least, injection of anti-insulin serum rapidly neutralizes biologically active insulin in the blood stream. The active hormone becomes again detectable when the injected serum has been saturated with bound insulin and then the blood-sugar concentration returns to normal levels. High concentrations of bound inactive insulin, however, continue to circulate in the blood for several days.

4. The relationship in different animals of endogenously secreted insulin to the insulin extracted from their pancreases is discussed.

We wish to thank Mr D. G. Taylor, F.R.C.S. of the Department of Thoracic Surgery, Guy's Hospital Medical School, who kindly carried out the experiments on the dogs; Miss C. N. Herbert who statistically analysed the results of the insulin assays; the Medical Research Council for a grant; Dr A. W. Stableforth and Professor R. H. S. Thompson for their encouragement; and Mrs E. B. Prince, Miss Anne Burton, Mr Kenneth Kilbourn and Mr D. Phelan for valuable technical assistance.

REFERENCES

ARMIN, J. & GRANT, R. T. (1953). The artery of the denervated rabbit's ear as a sensitive pharmacological test object. *J. Physiol.* **121**, 593–602.

ARMIN, J., GRANT, R. T. & WRIGHT, P. H. (1960*a*). Acute insulin deficiency provoked by single injections of anti-insulin serum. *J. Physiol.* **153**, 131–145.

ARMIN, J., GRANT, R. T. & WRIGHT, P. H. (1960*b*). Experimental diabetes in rats produced by parenteral administration of anti-insulin serum. *J. Physiol.* **153**, 146–165.

BAIRD, C. W. & BORNSTEIN, J. (1957). Plasma-insulin and insulin resistance. *Lancet*, **272**, 1111–1113.

BERSON, S. A. & YALOW, R. S. (1959). Species-specificity of human anti-beef, pork insulin serum. *J. clin. Invest.* **38**, 2017–2025.

GEY, G. O. & GEY, M. K. (1936). The maintenance of human normal cells and tumour cells in continuous culture. *Amer. J. Cancer*, **27**, 45–76.

HARRIS, J. I., SANGER, F. & NAUGHTON, M. A. (1956). Species differences in insulin. *Arch. Biochem. Biophys.* **65**, 427–438.

KING, E. J. (1951). *Microanalysis in Medical Biochemistry*, 2nd ed. London: Churchill.

MOLONEY, P. J. & COVAL, M. (1955). Antigenicity of Insulin: diabetes induced by specific antibodies. *Biochem. J.* **59**, 179–185.

MOLONEY, P. J. & GOLDSMITH, L. (1957). On the antigenicity of insulin. *Canad. J. Biochem. Physiol.* **35**, 79–92.

RANDLE, P. J. (1956). The assay of insulin in vitro by means of the glucose uptake of the isolated rat diaphragm. *J. Endocrin.* **14**, 82–86.

ROBINSON, B. H. B. & WRIGHT, P. H. (1961). Guinea-pig anti-insulin serum. *J. Physiol.* (in the Press).

SCOW, R. O. (1957). 'Total' pancreatectomy in the rat; operation, effects and post-operative care. *Endocrinology*, **60**, 359–367.

SOMOGYI, M. (1945). Determination of blood-sugar. *J. biol. Chem.* **160**, 69–73.

SOMOGYI, M. (1952). Notes on sugar determination. *J. biol. Chem.* **195**, 19–23.

VALLANCE-OWEN, J. & HURLOCK, B. (1954). Estimation of plasma-insulin by rat diaphragm method. *Lancet*, **266**, 68–70.

VALLANCE-OWEN, J. & WRIGHT, P. H. (1960). Assay of insulin in blood. *Physiol. Rev.* **40**, 219–244.

WRIGHT, P. H. (1957). Plasma-insulin estimation by the rat diaphragm method. *Lancet*, **273**, 621–624.

WRIGHT, P. H. (1959). The effect of insulin antibodies on glucose uptake by the isolated rat diaphragm. *Biochem. J.* **71**, 633–638.

WRIGHT, P. H. (1960*a*). Plasma-insulin activity in acromegaly and spontaneous hypoglycaemia. *Lancet*, **278**, 951–954.

WRIGHT, P. H. (1960*b*). Insulin antibodies. *Brit. med. Bull.* **16**, 219–223.

YALOW, R. S. & BERSON, S. A. (1960). Immuno-assay of plasma-insulin in man. *J. clin. Invest.* **39**, 1157–1175.

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THE EFFECT OF CARBACHOL ON THE PERMEABILITY OF DEPOLARIZED SMOOTH MUSCLE TO INORGANIC IONS

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Recent evidence, principally from electrical measurements, indicates that transmitter substances (e.g. acetylcholine) act at synapses by altering the permeability of the post-synaptic membrane to one or more inorganic ions. Such a mechanism was first proposed by Fatt & Katz (1951) to account for the depolarizing action of acetylcholine (ACh) at the end-plate region of skeletal muscle. The aim of the present work was to obtain more information about the nature of the change in permeability underlying the action of a stable analogue of ACh, carbachol, on mammalian smooth muscle. For this purpose, the movement of labelled inorganic ions was examined in preparations bathed in solutions containing sufficient potassium to depolarize the muscle fibres; the effects of carbachol on permeability could then be studied without the secondary changes in ion flux associated with the depolarization, and consequent increase in spike activity, which otherwise result from the action of the drug. A preliminary account of part of these results has been given to the Physiological Society (Durbin & Jenkinson, 1959).

METHODS

The taenia coli of the guinea-pig was used in these experiments, which were made at room temperature (20–23° C). The animal was killed by a blow on the head and the required amount of the taeniae excised immediately. It was found difficult to separate the muscle from the large intestine without removing at the same time some of the underlying tissue. Most of this was subsequently detached with fine scissors under a dissecting microscope. During dissection, the strips of taenia were bathed in a modified Krebs's solution which had the following composition (mM): NaCl 133, KCl 5·6, $NaHCO_3$ 16, $CaCl_2$ 2·5, $MgCl_2$ 1·15, glucose 6, Na phosphates 0·5, and was bubbled with a gas mixture containing 95 % O_2 and 5 % CO_2. After preparation, the strips were stretched to approximately their *in situ* length on frames of stainless-steel wire. They were then placed in a solution in which all but 5 mM of the NaCl had been replaced by K_2SO_4. It was found in preliminary experiments that immersion in potassium-rich fluids containing the full osmotic equivalent of the replaced NaCl caused the muscles to lose weight rather rapidly. For this reason, less K_2SO_4 was included in the potassium-rich solution finally employed, which had the following composition (mM): K_2SO_4 76, $KHCO_3$ 16, $CaCl_2$ 7·5, Na_2SO_4 2·5, $MgCl_2$ 1·15, glucose 6, Na

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phosphates 0·5. This was also bubbled with 95 % O_2 + 5 % CO_2 gas mixture, and was used throughout the present work, except in experiments with ^{82}Br, when 5 mM chloride was replaced by bromide. Strips of the taenia bathed in this solution maintained their weight, and responded reproducibly to carbachol, for many hours.

Efflux experiments. It was important for the present work that any net movements of ions should have been largely complete by the time of application of carbachol. The preparations were therefore bathed in the potassium-rich solution for several hours before commencing the experiment proper. During part of this equilibration period tracer was included, care being taken to ensure that active and inactive solutions were of the same chemical composition. In experiments with ^{42}K or ^{36}Cl, the preparations were loaded for 1–4 hr. Shorter loads (17–40 min) were employed in measurements with ^{82}Br.

In a number of experiments efflux of ^{42}K in exchange for inactive potassium was followed simultaneously with tension developed by the taenia. For this purpose the strip of muscle was mounted in a bath similar to that described by Born & Bülbring (1956). It consisted of a block of Perspex through which a horizontal channel, 4 cm long and 0·7 mm in diameter, had been drilled. One end of the strip was fixed at the beginning of the channel, the other being attached by silk thread to a mechano-electric transducer valve (RCA 5734), the output of which was fed, after amplification, to a pen recorder. An appropriate bathing solution flowed past the muscle at approximately 1 ml./min and was collected in bottles which were changed at intervals of 2–8 min. The amount of solution in each was determined by weighing, and 1 ml. samples taken for counting. At the end of the experiment, the activity of the muscle itself was determined, so that its tracer content at any earlier time could be established. To prepare the muscle for counting, it was either dissolved in 0·25 ml. of hot concentrated nitric acid, and the solution evaporated on a platinum planchette, or it was heated with 0·5 ml. of 0·2 N sodium hydroxide on a water bath for several hours, and the fluid finally transferred to a nickel planchette. The latter procedure was used in experiments with ^{36}Cl and ^{82}Br.

With ^{45}Ca and ^{36}Cl the specific activity of the tracer used was so low that it became necessary to use most of the taeniae coli of one guinea-pig for a single measurement. In such experiments the strips (of total weight from 60 to 120 mg) were tied to stainless-steel frames as before, and the loss of the isotope was followed by moving the pieces as a group into successive test-tubes containing known volumes (1·1–3 ml.) of inactive solution.

Uptake experiments. To study the effect of carbachol on the uptake of labelled ions, strips of the taeniae from one guinea-pig were first separated into groups, each containing pieces chosen so as to be similar in size and shape. After dissection each group was carefully blotted and weighed as a unit. The strips were then attached to frames and equilibrated in the potassium-rich solution for at least 2 hr. Subsequent procedures varied. In some experiments with ^{42}K two groups of strips were exposed for a short time, usually 7 min, to separate radioactive potassium-rich solutions, one of which contained carbachol. The muscles were then transferred to inactive fluid, and the amount of radioactivity which each group had acquired during the load period was determined in separate efflux measurements, as described in Results.

The foregoing procedure may involve an error if carbachol acts on the muscle membrane before the full specific activity of tracer has been reached in the immediate vicinity; a rather similar situation has been examined by Bianchi & Shanes (1959) in experiments to determine the effect of increases in the potassium concentration on the uptake of ^{45}Ca by skeletal muscle. Accordingly, in most experiments both groups of muscle strips were 'pre-loaded' in radioactive potassium-rich solution for 2–3 min before exposure separately to either another 'control' or to the 'carbachol' load solution.

Measurement of radioactivity. Throughout this work radioactivity has been determined by evaporating portions of active solutions in nickel or platinum planchettes and counting under an end-window G–M tube. The total weight of material on the planchette, including a disk of lens paper to promote even spreading, was held constant to avoid differences in

self-absorption. Count rates were corrected for background, which included that due to the ^{40}K of the potassium-rich solution used. An allowance for differences in back-scattering was made if nickel and platinum planchettes were used in the same experiment.

The isotopes used fell into two classes, the first having a short half-life and energetic radiation (^{24}Na, ^{42}K, ^{82}Br), the second a longer half-life and consisting of weak beta-emitters (^{45}Ca, ^{36}Cl). Two isotopes, one from each class, were used in many experiments. In such 'double-tracer' measurements, the activity of the short-lived isotope was first determined by counting through an aluminium absorber thick enough to stop beta radiation from the long-lived tracer. Later the sample was counted without absorber, after allowing sufficient time for the decay of the short-lived isotope. The success of the method depends on the freedom of the latter from long-lived radioactivity; several consignments of ^{42}K, supplied as K_2CO_3 by A.E.R.E., Harwell, were found, in fact, to contain appreciable amounts of such a contaminant (^{35}S).

RESULTS

Effect of carbachol on the efflux of potassium, bromide and chloride

Preliminary experiments were made to determine the time course of loss of labelled potassium, bromide and chloride from isolated taeniae coli

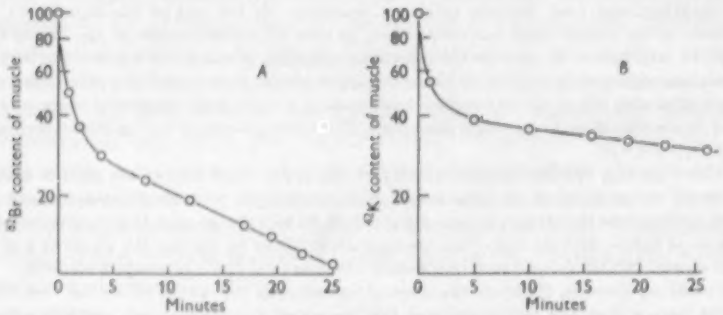

Fig. 1. Time course of loss of ^{82}Br (*A*) and ^{42}K (*B*) from separate portions of taenia coli bathed in inactive potassium-rich solution, at room temperature. Semilog. scales.

bathed in the potassium-rich solution used throughout the present work. Examples of the results obtained are illustrated in Fig. 1. Except for the first half-minute or so, the curves could be described as the sum of two exponential terms:

$$P = A\,e^{-\lambda_1 t} + B\,e^{-\lambda_2 t}, \qquad (1)$$

where P is the tracer content of the muscle at any time, t, and A, B, λ_1 and λ_2 are constants. Values obtained for λ_2 and, in a few cases, for λ_1 are listed in Table 1. After a few minutes the first term became unimportant, so that further loss of tracer followed a simple exponential law, as was found by Born & Bülbring (1956) for exchange of ^{42}K in taeniae coli bathed in Krebs's fluid at 37° C.

Carbachol consistently increased the rate of efflux, as is illustrated for ^{42}K in Figs. 2 and 3 (lower section). (In Fig. 3, and in some subsequent illustrations, the rate of loss has been plotted in terms of a coefficient, r, defined as the proportion of tracer lost in unit time, averaged over each collection period. r can be regarded as a convenient empirical measure of efflux, the use of which enabled results obtained with different isotopes to be expressed on a common basis. It may be noted that r approximates to λ_2 during the exponential phase of tracer loss.)

TABLE 1. Values of λ_1 and λ_2 (± S.E. of mean; no. of experiments in brackets) obtained in measurements of the rate of loss of ^{42}K, ^{36}Cl and ^{82}Br from the depolarized taenia coli, at room temperature

Ion	λ_1 (min^{-1})	λ_2 (min^{-1})
K	0·8, 0·7	0·012 ± 0·001 (9)
Cl		0·034 ± 0·002 (4)
Br	1·0, 1·0	0·040 ± 0·001 (5)

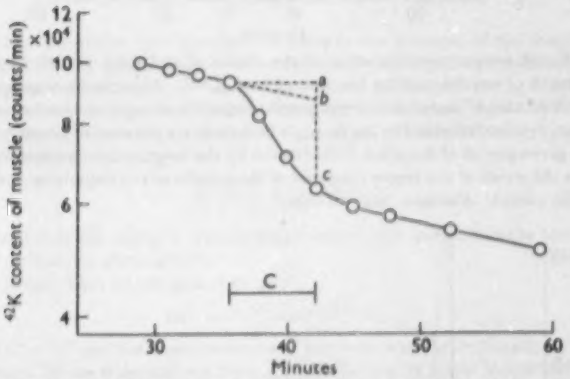

Fig. 2. Effect of 3×10^{-7} g/ml. carbachol (C) on loss of ^{42}K, plotted as in Fig. 1. A convenient measure of the action is given by the ratio, R_e, of the amounts of tracer represented by the distances *ac* and *ab* (see text). In this experiment R_e had the unusually large value of 4·9.

Carbachol also caused the muscle to develop additional tension (Fig. 3, upper section). This response to depolarizing agents applied in potassium-rich solutions has been described for other varieties of smooth muscle by Evans, Schild & Thesleff (1958), who also showed that, as in the polarized muscle, the contractures elicited by ACh were reversibly eliminated by atropine. An experiment to test this action on the depolarized taenia is illustrated in Fig. 4. It can be seen that atropine (3×10^{-8} g/ml.) almost abolished the increase in ^{42}K efflux produced by carbachol (3×10^{-7} g/ml.). The effect was only slowly reversible.

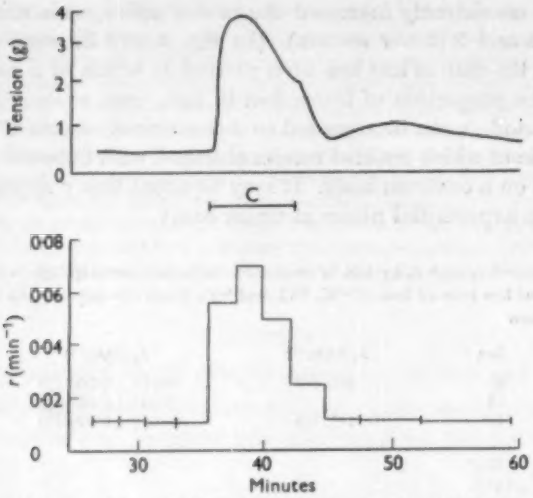

Fig. 3. Simultaneous determination of the effects of carbachol (3×10^{-7} g/ml.) on development of tension and on the rate of loss of ^{42}K. From same experiment as Fig. 2. Ordinates of upper and lower graphs respectively tension (grams) and rate coefficient, r (min^{-1}) defined as $\Delta c/\Delta t \cdot c_m$, where Δc is the amount of tracer collected during a given period of duration Δt (indicated by the length of the horizontal bars), and c_m is the mean of the tracer contents of the muscle at the beginning and at the end of the period. Abscissa, time in min.

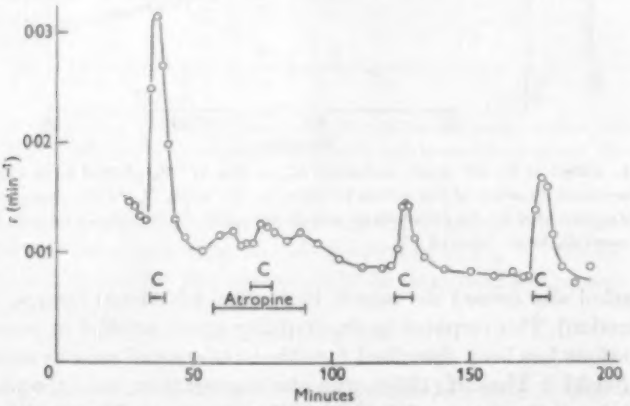

Fig. 4. Inhibition by atropine (3×10^{-6} g/ml.) of the increase in ^{42}K efflux produced by carbachol (3×10^{-7} g/ml.). Plotted as in lower part of Fig. 3, but with the rate of loss of tracer indicated for simplicity by points drawn at times corresponding to midway in each collection period. The additional tensions developed in response to the successive applications of the standard dose of carbachol were 2·9, 0·2, 0·3 and 0·8 g respectively.

To enable experiments of the kind illustrated in Figs. 2, 3 and 4 to be compared, carbachol was always applied in a concentration of 3×10^{-7} g/ml. for an arbitrary period of 7 min. A quantitative measure of the effect on ion flux was obtained by dividing the quantity of tracer lost during this standard application of the drug by the amount, estimated by extrapolation, which would presumably have exchanged had carbachol not been introduced (see Fig. 2). The average value of this ratio, R_e, obtained in nine measurements of the effect of the standard application of carbachol on ^{42}K efflux was $2{\cdot}32 \pm 0{\cdot}35$ (S.E. of the mean).

R_e may be expressed in terms of the rate constant, k, for exchange of tracer. To a first approximation, the loss of activity from the tissue is proportional to its tracer content, P:

$$-\frac{dP}{dt} = kP. \tag{2}$$

The application of carbachol results in an increase in the rate of loss (see Discussion), which may then be written

$$-\frac{dP}{dt} = (k + \Delta k)\, P, \tag{3}$$

where $k + \Delta k$ is a composite rate 'constant' holding in the presence of the drug. However, the effect of carbachol on a whole muscle develops slowly and is not well maintained (Fig. 3), possibly owing to desensitization of the receptors (Katz & Thesleff, 1957). For this reason, and to enable the effects of carbachol on both inward and outward fluxes to be compared, it is convenient to introduce a quantity $\overline{\Delta k}$, the average of Δk over the total time (from t to $t + \Delta t$) of application of the drug;

$$\overline{\Delta k} = \frac{1}{\Delta t}\int_t^{t+\Delta t} \Delta k\, dt. \tag{4}$$

It may be noted that $\overline{\Delta k}$, being a time-average value, will underestimate the maximum extent of the increase in permeability.

Equation (3) may then be integrated to give

$$\Delta P' = P'\{1 - e^{-(k+\overline{\Delta k})\,\Delta t}\}, \tag{5}$$

where P' and $P' - \Delta P'$ are the tracer contents of the muscle at the beginning and end of the carbachol period. If the drug had not been applied, the loss of tracer would have been ΔP, where

$$\Delta P = P'(1 - e^{-k\Delta t}). \tag{6}$$

Dividing (5) by (6), we obtain

$$\frac{\Delta P'}{\Delta P} = \frac{1 - e^{-(k+\overline{\Delta k})\,\Delta t}}{1 - e^{-k\Delta t}} = R_e, \text{ the efflux ratio as previously defined.} \tag{7}$$

Carbachol also increased the rate of loss of ^{36}Cl and ^{82}Br, as is illustrated in Fig. 5. R_e was found to be, for ^{36}Cl, $1{\cdot}33 \pm 0{\cdot}08$ (4 expts.) and for bromide, $1{\cdot}36 \pm 0{\cdot}05$ (3 expts.).

The effect of carbachol on uptake of potassium, bromide and chloride

If carbachol increases the permeability of the smooth muscle membrane, the rate of uptake of tracer from radioactive solutions should also be enhanced. This point was tested by briefly exposing two groups of strips

of taenia coli to separate load solutions which differed only in that one contained carbachol. The amount of tracer which had been taken up by each group was then determined, as illustrated in Fig. 6. The carbachol-treated muscles were invariably found to possess more radioactivity.

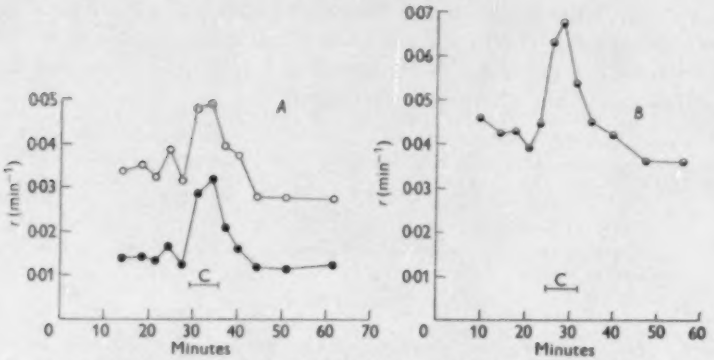

Fig. 5. The effect of carbachol (3×10^{-7} g/ml.) on the rate of loss of ^{36}Cl (○) and ^{42}K (●) (*A*, 'double tracer' technique employed), and ^{82}Br (◒) (*B*). Plotted as in Fig. 4.

The effect on uptake can be expressed in terms of the rate of exchange, k, just as in output experiments. For the control muscle, the rate of uptake of tracer is given by

$$\frac{dP}{dt} = k(P_t - P), \tag{8}$$

provided that the extracellular spaces have the same specific activity as the load solution. (P_t is the final steady state content of tracer, and other terms are as previously defined). Thus the amount of tracer, ΔP, taken up from time t to $t + \Delta t$ is

$$(P_t - P')\{1 - e^{-k\Delta t}\},$$

where P' is the activity of the muscle at time t.

The uptake, $\Delta P'$, by the carbachol-treated muscle during the same period is

$$(P_t - P')\{1 - e^{-(k+\overline{\Delta k})\Delta t}\},$$

where $\overline{\Delta k}$ is the time average of Δk, as before. Hence

$$\frac{\Delta P'}{\Delta P} = \frac{1 - e^{-(k+\overline{\Delta k})\Delta t}}{1 - e^{-k\Delta t}} = R_u, \tag{9}$$

the uptake ratio. Thus experimental determinations of R_u and R_e should provide similar values (cf. equation (7)).

In those experiments in which the muscles were 'pre-loaded' (see Methods) a correction was applied to allow for the fact that the carbachol-treated group were in contact with the drug only during 7 min of the total period (8–10 min) in load. It was assumed that, to a first approximation, the uptake of tracer was proportional to the load time; thus, for example, if the pre-loading period was 2 min, and the total time in load 9 min, 2/9 of the uptake by the control muscle was subtracted from the amounts of tracer taken up by the carbachol-treated group and by the control. The 'uptake ratio' was then expressed as the quotient of the differences.

In six measurements of the effect of the standard application of carbachol on the uptake of ^{42}K, the mean value obtained for R_u was $1{\cdot}88 \pm 0{\cdot}12$. (In one of these experiments the muscles had been 'pre-loaded', and in another, three groups of muscles were used, to check the validity of the correction described in the preceding paragraph; the values obtained were respectively 2·29 and 2·00, and have been included with the others.) In the remaining uptake experiments to be described, the 'pre-loading' technique was employed.

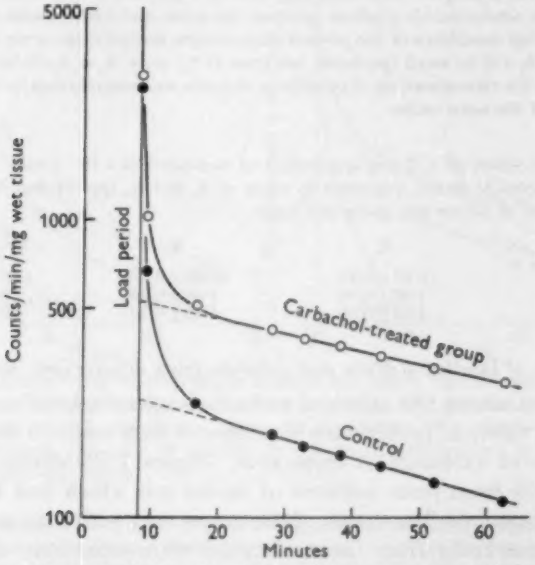

Fig. 6. The effect of carbachol on the uptake of ^{42}K. Two portions of the taenia have been exposed (without pre-loading, in this case) to separate load solutions, one of which contained carbachol (3×10^{-7} g/ml.). After 8 min both groups were transferred to inactive fluid and the loss of tracer was followed; the quantity remaining in each is shown as a function of time (cf. Fig. 1). The amounts which had been taken up can be compared by extrapolating the linear parts of the curves to time of removal from the load, which contained ^{42}K $2{\cdot}9 \times 10^4$ counts/min per μmole K. Semi-log. scale.

Carbachol also increased the influx of labelled chloride and bromide. In four experiments with ^{36}Cl, R_u came to $1{\cdot}38 \pm 0{\cdot}10$, and in three with ^{82}Br, $1{\cdot}28 \pm 0{\cdot}10$. These figures, together with the corresponding efflux ratios, are summarized in Table 2. It can be seen that for a given ion, R_e and R_u agree within the error of the measurements. Table 2 also lists values for $\overline{\Delta k}$, calculated, by equation (7), from the mean of R_u and R_e for

each isotope. The figures obtained are also in fair agreement. The possible significance of this is discussed later.

It has been assumed in applying equation (7) to the experimental data that k, the rate constant for exchange of tracer, is given by λ_3 (equation (1)). However, the two terms of equation (1) correspond to loss of tracer from interspaces and cells respectively only if these regions can be considered as being in independent equilibrium with the bathing fluid. In practice, it is likely that tracer from a proportion of the cells can gain access to the exterior only through the extracellular spaces, which thus have an appreciable specific activity. It can be shown that λ_3 may then differ substantially from k, and A and B from the original tracer contents of interspaces and cells, respectively, especially if the tissue is thick and if there is a large concentration gradient between the intra- and extracellular fluids (Harris, 1950). Under the conditions of the present experiments, however, the error introduced by equating k to λ_3 will be small (probably less than 10%) since $\lambda_2 \ll \lambda_1$ (Table 1), and since it is likely that the concentrations of potassium, chloride and bromide ions in the interspaces and cells are of the same order.

TABLE 2. The effect of a 7 min application of carbachol (3×10^{-7} g/ml.) on potassium, chloride and bromide fluxes, expressed in terms of R_i and R_o (pp. 79 and 80). The corresponding values of $\overline{\Delta k}$ are also given (see text)

Ion	R_i	R_o	$\overline{\Delta k}$ (min^{-1})
K	$2{\cdot}32 \pm 0{\cdot}35$	$1{\cdot}88 \pm 0{\cdot}12$	0·014
Cl	$1{\cdot}33 \pm 0{\cdot}08$	$1{\cdot}38 \pm 0{\cdot}10$	0·014
Br	$1{\cdot}36 \pm 0{\cdot}05$	$1{\cdot}28 \pm 0{\cdot}10$	0·015

Loss of labelled sodium and calcium from depolarized taeniae

Before examining the effect of carbachol on the rate of movement of sodium and calcium, preliminary experiments were made to determine the time course of exchange of these ions. Figure 7 illustrates the loss of ^{24}Na and ^{45}Ca from three portions of taenia coli which had been loaded for different periods. The shapes of the curves vary with the time of loading and differ markedly from those observed with potassium, chloride and bromide (cf. Fig. 1).

Except for the first half-minute or so, the loss of tracer could be described as the sum of three exponential terms. In Fig. 7 the uppermost curves, which refer to the longest load times, have been drawn according to the expression

$$P = A\,e^{-\lambda_1 t} + B\,e^{-\lambda_2 t} + C\,e^{-\lambda_3 t}, \qquad (10)$$

where P is the tracer content of the muscle at any time, and A, B and C have the values (in counts/min/mg muscle) of 575, 360 and 11 (^{24}Na) and 150, 67 and 18 (^{45}Ca). The corresponding values of λ_1, λ_2 and λ_3 were 0·26, 0·086 and 0·0097 min^{-1}, for loss of ^{24}Na, and 0·21, 0·067 and 0·0061 min^{-1} for loss of ^{45}Ca. It was of some interest to compare values obtained for A in experiments with sodium and calcium on the one hand, and with potassium, chloride and bromide on the other. In the experiment of Fig. 7 the activity of the load solution was $1{\cdot}34 \times 10^6$ counts/min/ml. (^{24}Na) and

$4{\cdot}28 \times 10^5$ counts/min/ml. (^{45}Ca), so that the values of A corresponded to volumes which were respectively 43 and 35 ml./100 g wet tissue. These figures, and others obtained in measurements of this kind, are similar to the values observed in experiments with labelled potassium, chloride and bromide (Table 3). It thus seems reasonable to identify the first term in equation (10) with loss of tracer from the interspaces.

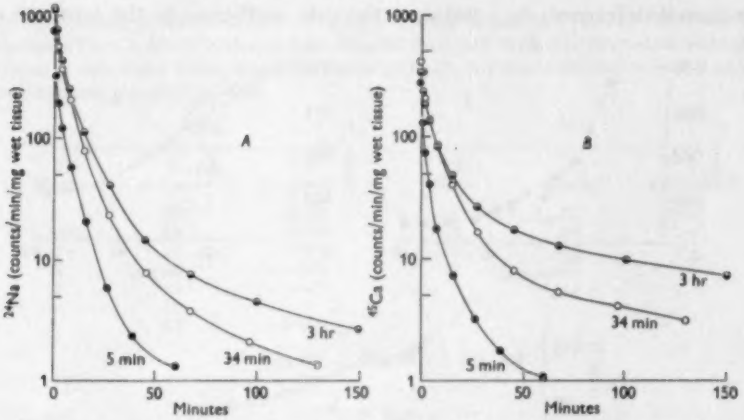

Fig. 7. 'Double tracer' experiment to demonstrate loss of ^{24}Na (A) and ^{45}Ca (B) from three portions of taenia from the same guinea-pig. The duration of the load period is given with each curve. $2{\cdot}35 \times 10^5$ counts/min ^{24}Na corresponded to 1μ-mole sodium, and $5{\cdot}7 \times 10^4$ counts/min ^{45}Ca to 1μ-mole calcium in the load solution. Semi-log. scales.

TABLE 3. Values for A, and in a few cases for B, obtained on fitting equation (10) to the efflux of different labelled ions. Both quantities have been expressed as the equivalent volumes of load solution (ml./100 g wet tissue). A value for B has been given only if the load period exceeded 2 hr (^{36}Cl) or 3 hr (^{42}K)

Isotope	A	B	Isotope	A
^{42}K	59, 39	32, 41, 53, 37	^{45}Ca	44, 32, 46, 44
^{36}Cl	43, 42	31, 47	^{24}Na	37, 44
^{82}Br	41, 39	—	—	—

It can be seen from Fig. 7 that the tracer content of muscles which had been loaded for as long as 3 hr fell to less than 5% of the original value after only 1 hr in inactive solution. Thereafter activity was lost much more slowly, indicating that part of the sodium and calcium in the muscle is relatively inexchangeable under the present conditions. Because this component itself may well be non-homogeneous, the second and third terms in equation (10) cannot be taken to correspond with any certainty to loss of tracer from distinct compartments or fractions.

Effect of carbachol on efflux of sodium and calcium

The results of experiments to test the action of carbachol on the rate of loss of ^{24}Na and ^{45}Ca are illustrated in Fig. 8. The effects observed were small and rather irreproducible. Because of the complexity of the efflux curves, no attempts were made to compute values for the efflux ratio, R_e, as previously defined. Instead, the actions were expressed as the maximum difference, Δr_m, between the rate coefficient in the presence of

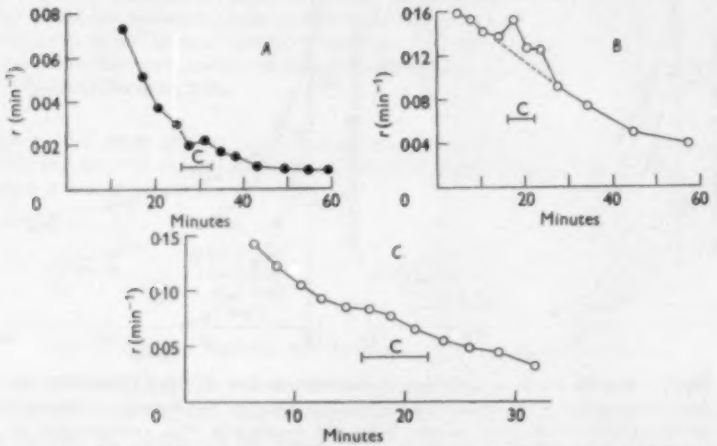

Fig. 8. Effect of carbachol (3×10^{-7} g/ml.) on the efflux of ^{45}Ca (●, *A*) and ^{24}Na (○, *B* and *C*) from depolarized taenia coli. Plotted as in Fig. 4.

carbachol, and that, r, which would have held had the drug not been applied; e.g. in the experiment of Fig. 8(*B*), Δr_m came to

$$0{\cdot}15\text{–}0{\cdot}12 = 0{\cdot}03 \text{ min}^{-1}.$$

This value is listed in Table 4 together with others obtained in a series of experiments in which carbachol was applied at different times after removal of the taenia from the load solution, in an attempt to determine whether a particular phase of tracer exchange was affected by the drug. The results suggest that this was not the case.

Effect of carbachol on the uptake of sodium and calcium

Figure 9 illustrates a typical experiment to test the action of carbachol on the uptake of ^{24}Na and ^{45}Ca by taenia coli. Because of the complex time course of exchange of these ions, it is difficult to make a direct comparison of the amounts of tracer taken up by the cellular material of the test and control muscles during the load period. Instead, the ratio of the total

tracer contents (per milligram wet tissue) of the two groups was computed for different intervals after the transfer of both from active to inactive solution. The mean values obtained in all experiments of this kind are shown plotted in Fig. 10. It can be seen that the carbachol-treated muscles contained more tracer than did the controls, from which it can be concluded that the drug affects the uptake of these ions.

TABLE 4. Effect of carbachol (3×10^{-7} g/ml.) on rate of loss of ^{24}Na and ^{45}Ca from the depolarized taenia coli. The time, t, from removal from load to the application of carbachol is listed in cols. 2 and 5 and the maximum increase, Δr_m, in rate coefficient in cols. 3 and 6. A — indicates a negligible effect

	^{24}Na			^{45}Ca	
1 Expt.	2 t (min)	3 Δr_m (min^{-1})	4 Expt.	5 t (min)	6 Δr_m (min^{-1})
1	15	0·015	1	15	—
2*	16	0·030	2	16	—
3†	17	0·007	8‡	26	—
4	18	0·009	9	33	—
5	21	—			
6	46	—			
7	58	0·005			

* Fig. 8*B*; † Fig. 8*C*; ‡ Fig. 8*A*.

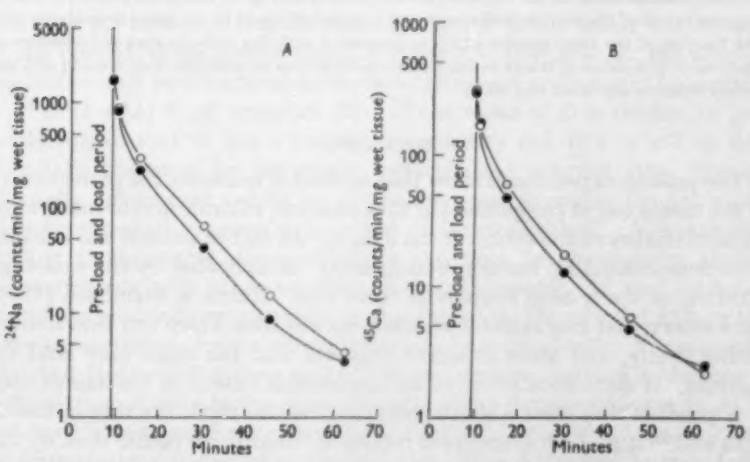

Fig. 9. 'Double tracer' experiment to test the effect of carbachol (3×10^{-7} g/ml.) on the uptake of ^{24}Na (*A*) and ^{45}Ca (*B*). Plotted as in Fig. 6. $4{\cdot}20 \times 10^5$ counts/min ^{24}Na correspond to 1μ-mole sodium, and $4{\cdot}10 \times 10^4$ counts/min ^{45}Ca to 1μ-mole calcium; it should be noted that in this early experiment the total calcium concentration was maintained at 10 mM. The carbachol-treated muscles (○) have taken up a slightly greater amount of both tracers than have the controls (●). Semi-log scales.

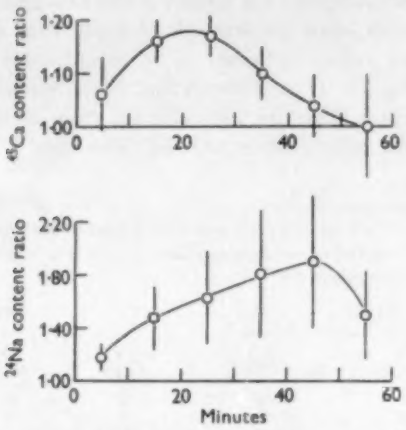

Fig. 10. Combined results of experiments (10 with ^{24}Na, 6 with ^{45}Ca) of the type illustrated in Fig. 9. The tracer contents of the carbachol-treated muscles have been divided by that of the controls, for various times after removal from load. Mean values, ± S.E. of means, plotted.

The initial increases in the 'content ratios' plotted in Fig. 10 are most probably due to progressive loss of tracer from the interspaces. Carbachol would be expected to enhance only that fraction of the total uptake which is associated with the cells, so that the presence of appreciable quantities of tracer in the extracellular spaces of both the drug-treated and the control muscles will mask the effect.

DISCUSSION

The present experiments show that carbachol increases the permeability of the taenia coli of the guinea-pig to potassium, chloride and bromide ions. Parallel studies of the action of the drug on the flux of sodium and calcium were complicated by the non-homogeneity, as indicated by the exchange kinetics, of the muscle content of these ions. Harris & Steinbach (1956) have shown that frog skeletal muscle possesses sites which can bind sodium rather firmly, and other evidence suggests that the same may hold for calcium. If such sites occur to an appreciable extent in the taenia coli, it is possible that after a short period in inactive fluid, the rate of loss of ^{24}Na and ^{45}Ca may be determined largely by desorption rather than by the permeability of the membrane. The failure to observe appreciable effects of carbachol on the efflux would not then be unexpected. However, this should not apply to the study of inward movement and in fact the uptakes of both sodium and calcium were enhanced by the drug, suggesting, although in a less conclusive manner, that the permeability to these ions is also increased by carbachol.

The analysis made by Fatt & Katz (1951) of the end-plate potential of frog skeletal muscle showed that ACh acts as though it created additional pathways for the movement of ions through the post-synaptic region. These would appear to differ from the normal channels in at least two respects; they permit the comparatively free passage of sodium, potassium and possibly some other ions (but not apparently of chloride (Takeuchi & Takeuchi, 1960); however, see Nastuk (1959)), and they allow externally-applied current to pass in both directions with much the same facility under circumstances in which the conducting channels normally present in the membrane show marked 'anomalous rectification' (del Castillo & Katz, 1955). This suggests that the ACh channels may be 'in parallel' with the others. It is of interest to express the present findings in terms of such a model. In all the experiments which have been described, the membrane was already depolarized by the application of potassium-rich fluids. It is then unlikely that the action of carbachol can give rise to an appreciable change in membrane potential, so that the additional ion flux produced by the drug might be expected to follow Fick's law, and so could be written as

$$\frac{\Delta A \,.\, D}{V \,.\, x} P,$$

where D is the effective diffusion coefficient in the carbachol-operated channels (of area ΔA, and thickness x), and P is the tracer content and V the volume, of the muscle fibres. Writing $\Delta A \,.\, D/V \,.\, x$ as Δk, the action of carbachol may be considered to increase the rate of exchange of tracer from $k \,.\, P$ to $(k+\Delta k)\, P$ (cf. equation (3)). If the value of D is similar, or proportional, to that in free solution, the quantity $\Delta A \,.\, D/V \,.\, x$ will be substantially the same for potassium, chloride and bromide ions. Experimentally determined values for Δk, and so most probably for $\overline{\Delta k}$, would then be expected to agree, as is in fact observed (Table 2). Although this finding is suggestive, its significance can only be decided by similar measurements with other varieties of smooth muscle. It may be noted that there is already much evidence to suggest that the effect of ACh on the ionic permeability of some other tissues may be more specific. For example, Harris & Hutter (1956) have demonstrated a striking increase in the rate of potassium exchange in the sinus venosus of the frog in response to ACh, but have not observed any comparable effect on the flux of sodium, chloride or bromide ions (personal communication). This difference in specificity most probably reflects the *inhibitory* action of ACh on the sinus (cf. Hutter, 1957).

Even when depolarized by immersion in potassium-rich solution, the taenia coli develops additional tension on the application of carbachol. The possibility arises that the observed changes in ion flux may have been

artifacts of the mechanical response, for example, due to 'squeezing out' of tracer from the extracellular spaces. However, the finding that inward and outward fluxes were increased to approximately the same extent suggests that this was not the case. Also, it was possible, by making a suitable reduction in the calcium concentration, to observe large effects of carbachol on ion movement in the almost complete absence of tension development. These experiments, which were made to obtain more information about the mechanism of the contracture, are described more fully in the following paper (Durbin & Jenkinson, 1961).

SUMMARY

1. Radio-isotopes have been used to study the effect of carbachol on the permeability of the taenia coli of the guinea-pig to inorganic ions. The preparations were bathed in potassium-rich solutions throughout in order to avoid secondary ionic movements associated with the changes in membrane potential which otherwise accompany the action of the drug.

2. Carbachol increased both inward and outward fluxes of potassium, chloride and bromide ions, and caused the muscle to develop additional tension. Both responses were reversibly abolished by atropine (3×10^{-8} g/ml.).

3. Similar experiments on ^{24}Na and ^{45}Ca flux were complicated by the relative inexchangeability of part of the muscle sodium and calcium. However, the results suggested that carbachol also affects the movement of these ions.

4. These findings support the hypothesis that depolarizing agents such as carbachol act by increasing the permeability of the muscle membrane to inorganic ions.

We are indebted to Professor B. Katz for constant help and encouragement, and to Mr J. L. Parkinson, Miss A. Paintin and Mr L. Ward for frequent assistance.

REFERENCES

Bianchi, C. P. & Shanes, A. M. (1959). Calcium influx in skeletal muscle at rest, during activity, and during potassium contracture. *J. gen. Physiol.* **42**, 803–815.

Born, G. V. R. & Bülbring, E. (1956). The movement of potassium between smooth muscle and the surrounding fluid. *J. Physiol.* **131**, 690–703.

del Castillo, J. & Katz, B. (1955). Local activity at a depolarized nerve-muscle junction. *J. Physiol.* **128**, 396–411.

Durbin, R. P. & Jenkinson, D. H. (1959). Effect of carbachol on potassium and chloride flux in depolarized smooth muscle. *J. Physiol.* **148**, 68–69*P*.

Durbin, R. P. & Jenkinson, D. H. (1961). The calcium dependence of tension development in depolarized smooth muscle. *J. Physiol.* **157**, 90–96.

Evans, D. H. L., Schild, H. O. & Thesleff, S. (1958). Effects of drugs on depolarized plain muscle. *J. Physiol.* **143**, 474–485.

Fatt, P. & Katz, B. (1951). An analysis of the end-plate potential recorded with an intracellular electrode. *J. Physiol.* **115**, 320–370.

HARRIS, E. J. (1950). The transfer of sodium and potassium between muscle and the surrounding medium. Part II. The sodium flux. *Trans. Faraday Soc.* **46**, 872–882.

HARRIS, E. J. & HUTTER, O. F. (1956). The action of acetylcholine on the movements of potassium ions in the sinus venosus of the heart. *J. Physiol.* **133**, 58–59*P*.

HARRIS, E. J. & STEINBACH, H. B. (1956). The extraction of ions from muscle by water and sugar solutions with a study of the degree of exchange with tracer of the sodium and potassium in the extracts. *J. Physiol.* **133**, 385–401.

HUTTER, O. F. (1957). Mode of action of autonomic transmitters on the heart. *Brit. med. Bull.* **13**, 176–180.

KATZ, B. & THESLEFF, S. (1957). A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *J. Physiol.* **138**, 63–80.

NASTUK, W. L. (1959). Some ionic factors that influence the action of acetylcholine at the muscle end-plate membrane. *Ann. N.Y. Acad. Sci.* **81**, 317–327.

TAKEUCHI, A. & TAKEUCHI, N. (1960). On the permeability of the end-plate membrane during the action of transmitter. *J. Physiol.* **154**, 52–67.

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THE CALCIUM DEPENDENCE OF TENSION DEVELOPMENT IN DEPOLARIZED SMOOTH MUSCLE

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(*Received* 28 *November* 1960)

It is known that several types of smooth muscle contract on exposure to acetylcholine (ACh), even when depolarized by immersion in isotonic solutions of potassium chloride or sulphate (Evans, Schild & Thesleff, 1958). The present study originated from the finding that the magnitude of this response was dependent on the calcium content of the bathing fluid. It seemed possible that this might provide a clue to the mechanism of the contracture, for there is evidence to suggest that tension development in the heart (Niedergerke, 1956; Lüttgau & Niedergerke, 1958), and possibly in skeletal muscle (Bianchi & Shanes, 1959; Frank, 1960) is initiated by additional entry of calcium into the cells. If smooth muscle is similar in this respect, it is conceivable that the ACh contracture is a direct consequence of the action of the drug in increasing the permeability of the membrane to calcium (Durbin & Jenkinson, 1961). It thus seemed of interest to examine the effects of a reduction in the calcium concentration on the magnitude of (*a*) the contracture and (*b*) the simultaneous increase in membrane permeability which follow the application of a stable analogue of ACh, carbachol, to a smooth muscle bathed in potassium-rich solution.

METHODS

The experiments were made at room temperature (19–23° C) with isolated portions of the taenia coli of the guinea-pig. For measurements of the rate of potassium exchange a strip of muscle was attached to a stainless-steel frame and exposed for several hours to a solution containing ^{42}K. It was then transferred to a continuously flowing stream of inactive fluid and the loss of tracer followed; the tension developed by the strip was recorded simultaneously by means of a transducer valve coupled to a pen recorder. Throughout such experiments the preparations were bathed in solutions containing for the most part potassium sulphate (76 mM) and potassium bicarbonate (16 mM). It should be noted that a considerable part of the total concentration (7·5 mM) of calcium normally included may have been in a combined form, because of binding by carbonate and by sulphate (Hill & Howarth, 1957; Hodgkin & Horowicz, 1959). Further details of the composition of this and other solutions used, and of the procedure in tracer experiments, have been given in the preceding paper (Durbin & Jenkinson, 1961).

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RESULTS

The first experiments were made to determine the calcium dependence of the response of the taenia coli to prolonged depolarization by application of the potassium-rich solution used in much of the present work. A typical 'potassium contracture' is illustrated in Fig. 1. It may be seen that the tension first rose rapidly, and then fell more slowly to an inter-

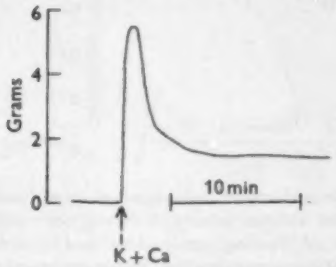

Fig. 1. Mechanical response of the taenia coli to replacement of the Krebs's bathing fluid by a solution containing 168 mM potassium and 7·5 mM calcium. Isometric recording at room temperature.

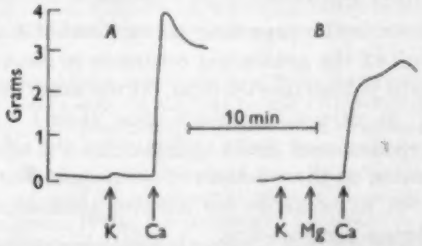

Fig. 2. Calcium dependence of 'potassium contracture'. In *A*, the muscle strip had been bathed for 15 min in calcium-free Krebs's fluid. At first arrow, this was replaced by calcium-free potassium-rich solution. At second arrow, calcium chloride (7·5 mM) was added. In *B*, obtained using another strip, the reintroduction of calcium was preceded by the addition of 30 mM magnesium chloride.

mediate level, hereafter referred to as 'resting' tension. This rarely declined further, and with some preparations tended to increase over a period of several hours. Its value was not affected by omission of the 5·7 mM sodium normally included in the potassium-rich fluid.

On repeating the experiment of Fig. 1, but with Ca-free solutions, it was found that the 'potassium contracture' did not then occur. A similar observation has been reported for frog skeletal muscle (Denton, 1948; Frank, 1960), for the heart (Niedergerke, 1956) and, most recently, for rat uterine muscle (Edman & Schild, 1961). Restoration of calcium, but not of

magnesium, was followed by an immediate contracture, as illustrated in Fig. 2. As might be expected, subsequent alterations in the calcium concentration produced corresponding changes in 'resting' tension (Fig. 3*a* and *b*).

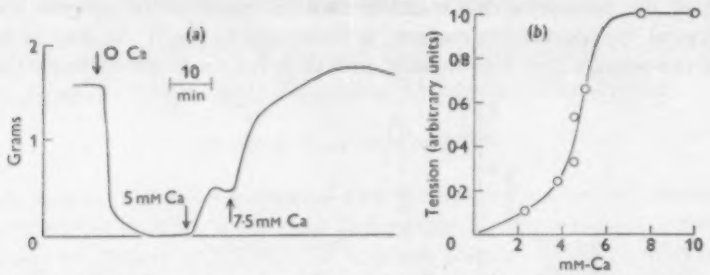

Fig. 3. (*a*) Calcium dependence of maintained phase of 'potassium contracture'. Bathing fluid contained 168 mM potassium throughout; initial and final calcium concentration, 7·5 mM. (*b*) 'Resting' tension developed by another strip of the taenia coli as a function of calcium concentration. Values expressed as fractions of that in a potassium-rich fluid containing 7·5 mM calcium.

Calcium dependence of the response to carbachol

It has been shown in the preceding paper (Durbin & Jenkinson, 1961) that the taenia coli of the guinea-pig contracts in response to carbachol even when bathed in potassium-rich fluid; at the same time, an increase in the permeability to certain inorganic ions occurs. The experiments described in this section were made to determine the effects of changes in calcium concentration on these actions of carbachol. For this purpose the rate of efflux of ^{42}K in exchange for inactive potassium was taken as a measure of membrane permeability.

A first experiment showed that 15 min after the introduction of a calcium-free solution both the increase in tension, and less expectedly, the change in permeability produced by a standard application of carbachol, had fallen reversibly to less than 5% of the control values. The effects of intermediate reductions in the calcium content of the bathing fluid were then tested, as illustrated in Fig. 4, where it may be seen that there was little mechanical response to carbachol applied in a solution containing one fifth of the previous calcium concentration, although an appreciable increase in the rate of efflux of ^{42}K still occurred. The results of this and four other such experiments are listed in Table 1. Although the sensitivity to calcium varied considerably from preparation to preparation, the reduction in the contracture tension exceeded that in the change in permeability produced by carbachol in every case.

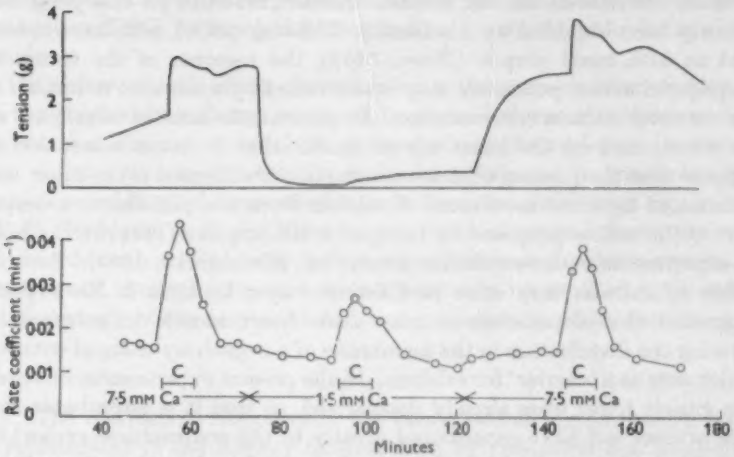

Fig. 4. Effect of a reduction (from 7·5 to 1·5 mM) in calcium concentration on the increases in tension and in rate of exchange of labelled potassium produced by a standard application of carbachol (3×10^{-7} g/ml. for 7 min). Potassium concentration 168 mM throughout; tonicity of low calcium solutions maintained with sucrose. Upper and lower ordinates, respectively, isometric tension (g), and rate of loss of ^{42}K, measured as described in preceding paper (Durbin & Jenkinson, 1961).

TABLE 1. Summary of five experiments of the type illustrated in Fig. 4. In each, a strip of taenia coli previously loaded with ^{42}K has been bathed successively in inactive potassium-rich solutions containing the concentrations of calcium listed in columns 1, 2 and 3. Carbachol (3×10^{-7}g/ml.) was applied in each solution, and the corresponding tension responses are listed in columns 4, 5 and 6 respectively. Columns 7, 8 and 9 give the simultaneously measured increases in the rate of loss of ^{42}K, expressed in terms of R_e, as defined in the preceding paper (Durbin & Jenkinson, 1961). For comparison, the effects on tension and ^{42}K flux produced by the standard concentration of carbachol when applied in low calcium solutions have been expressed as percentages of the mean values of the controls in columns 10 and 11 respectively

	1	2	3	4	5	6	7	8	9	10	11
Expt.	[Ca] (mM)			ΔT (g)			R_e			% tension	% flux
1	3·5	0·70	7·0	0·7	0·1	0·7	1·7	1·3	1·9	14	72
2	7·5	0·20	7·5	2·9	0·25	2·7	2·0	0·5	2·6	9	22
3	7·5	0·85	7·5	3·5	0·05	3·2	5·1	1·7	5·0	1·5	34
4*	7·5	1·50	7·5	1·25	0·20	1·2	2·2	1·6	2·3	16	71
5	7·5	0·23	7·5	3·15	0·15	2·8	2·2	0·95	2·7	5	39

* Fig. 4.

DISCUSSION

The necessity of calcium for 'potassium contractures' of the taenia coli of the guinea-pig, and of the uterus of the rat (Edman & Schild, 1961), suggests that calcium plays an important part in the mechanical response

of these varieties of smooth muscle. Further evidence on this point has recently been obtained by Axelsson & Bülbring (1959), who have shown that as with heart muscle (Mines, 1913), the response of the taenia to propagated action potentials may be abolished by a suitable reduction in the external calcium concentration. In general, the actions of calcium on the taenia and on the heart are so similar that it seems reasonable to suppose that the process whereby contraction is activated is the same, and is initiated by a net movement of calcium from a superficial to a deeper part of the cell, as proposed by Lüttgau & Niedergerke (1958) on the basis of experiments with ventricular muscle (cf. Niedergerke, 1959). Such an influx of calcium may arise in different ways; Lüttgau & Niedergerke suggested that depolarization may cause heart muscle to contract by altering the distribution in the membrane of a negatively charged complex which acts as a 'carrier' for calcium. In the present experiments, however, the muscle fibres were already depolarized, so that it is improbable that this process can have contributed greatly to the contractures evoked by carbachol. A more likely explanation is to be found in the action of the drug in increasing the permeability of the membrane to calcium. This would give rise to a net influx, and so presumably initiate contraction, provided that a concentration gradient existed across the membrane. Although direct evidence for a low internal concentration of unbound calcium has so far been obtained with only one excitable tissue, the giant axon of the squid (Hodgkin & Keynes, 1957), it seems likely that this may be a rather general phenomenon; Gilbert & Fenn (1957) have shown that skeletal muscle can maintain the intracellular calcium concentration against an electrochemical gradient, probably by 'active transport'.

On this view the size of the carbachol contracture might be expected to vary with (*a*) the concentration gradient for calcium and (*b*) the extent of the increase in permeability. The finding that the effect of carbachol on ion flux is itself calcium dependent suggests that reductions in both (*a*) and (*b*) contribute to the failure of the mechanical response which occurs in solutions containing little calcium. However, the fall in (*a*) appears to be the more important factor, for it was possible, on suitably lowering the calcium concentration, to observe a relatively large action of carbachol on permeability, in the almost complete absence of tension development. It had been hoped to obtain more exact information on this point by using ^{45}Ca to make direct measurements of the effect of carbachol on the movement of calcium. Although the expected increase in uptake was observed, as described in the preceding paper (Durbin & Jenkinson, 1961), the complexity of the kinetics of exchange in this tissue made it difficult to study the effect quantitatively. It is possible that other varieties of smooth muscle may prove more satisfactory for this purpose; Robertson (1960)

has reported in a preliminary communication that ACh markedly increases the uptake of ^{45}Ca by depolarized longitudinal muscle from the ileum of the rabbit.

The rather variable effect of a reduction in the concentration of calcium on the permeability change produced by carbachol was of interest as it is known that the actions of ACh in depolarizing the end-plate region of frog skeletal muscle (del Castillo & Stark, 1952), and in increasing the ionic permeability of denervated muscle from the diaphragm of the rat (D. H. Jenkinson & J. G. Nicholls, unpublished observations), are not calcium dependent. This may reflect some difference in the receptors of smooth and skeletal muscle, but this can only be decided by a more detailed study of the actions of ACh and carbachol on these tissues.

SUMMARY

1. The mechanical responses of the taenia coli of the guinea-pig both to replacement of the Krebs's bathing fluid by potassium-rich solutions, and to subsequent applications of carbachol, are abolished in the absence of calcium.

2. Carbachol becomes less effective in increasing the membrane permeability of the depolarized taenia when the calcium content of the bathing fluid is lowered. However, over a certain range of calcium concentrations, the reduction in the accompanying contracture is greater, so that the two actions of the drug can be almost dissociated.

3. These findings are discussed in relation to the hypothesis that the carbachol contracture of depolarized smooth muscle is a consequence of net movement of calcium into the cells, following the increase in permeability produced by the drug.

We are indebted to Professor B. Katz for constant help and encouragement and to Mr J. L. Parkinson, Miss A. Paintin and Mr L. Ward for frequent assistance.

REFERENCES

Axelsson, J. & Bülbring, E. (1959). Some means of abolishing the tension response in smooth muscle during continued electrical activity at the cell membrane. *J. Physiol.* **149**, 50–51*P*.

Bianchi, C. P. & Shanes, A. M. (1959). Calcium influx in skeletal muscle at rest, during activity, and during potassium contracture. *J. gen. Physiol.* **42**, 803–815.

del Castillo, J. & Stark, L. (1952). The effect of calcium ions on the motor end-plate potentials. *J. Physiol.* **116**, 507–515.

Denton, E. J. (1948). The role of calcium in contractures of the rectus abdominis of the frog. *J. Physiol.* **107**, 32*P*.

Durbin, R. P. & Jenkinson, D. H. (1961). The effect of carbachol on the permeability of depolarized smooth muscle to inorganic ions. *J. Physiol.* **157**, 74–89.

Edman, K. A. P. & Schild, H. O. (1961). Interactions of acetylcholine, adrenaline and magnesium with calcium in the contraction of depolarized rat uterus. *J. Physiol.* **155**, 10–11*P*.

EVANS, D. H. L., SCHILD, H. O. & THESLEFF, S. (1958). Effects of drugs on depolarized plain muscle. *J. Physiol.* **143**, 474–485.

FRANK, G. B. (1960). Effects of changes in extracellular calcium concentration on the potassium-induced contracture of frog's skeletal muscle. *J. Physiol.* **151**, 518–538.

GILBERT, D. L. & FENN, W. O. (1957). Calcium equilibrium in muscle. *J. gen. Physiol.* **40**, 393–408.

HILL, A. V. & HOWARTH, J. V. (1957). The effect of potassium on the resting metabolism of the frog's sartorius. *Proc. Roy. Soc.* B, **147**, 21–43.

HODGKIN, A. L. & HOROWICZ, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* **148**, 127–160.

HODGKIN, A. L. & KEYNES, R. D. (1957). Movement of labelled calcium in squid giant axons. *J. Physiol.* **138**, 253–281.

LÜTTGAU, H. C. & NIEDERGERKE, R. (1958). The antagonism between Ca and Na ions on the frog's heart. *J. Physiol.* **143**, 486–505.

MINES, G. R. (1913). On functional analysis by the action of electrolytes. *J. Physiol.* **46**, 188–235.

NIEDERGERKE, R. (1956). The potassium chloride contracture of the heart and its modification by calcium. *J. Physiol.* **134**, 584–599.

NIEDERGERKE, R. (1959). Calcium and the activation of contraction. *Experientia*, **15**, 128–130.

ROBERTSON, P. A. (1960). Calcium and contractility in depolarized smooth muscle. *Nature, Lond.*, **186**, 316–317.

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THE SELECTIVE APPETITE FOR Na^+ SHOWN BY Na^+-DEFICIENT SHEEP

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(*Received 5 December* 1960)

In view of the importance of Na^+ equilibrium to normal function of higher species, an innate behaviour pattern promoting its intake in the face of deficiency could give an important survival advantage to the organism. Richter (1956) has shown that young rats, for whom their mother's milk has been the sole source of nourishment, consistently showed an active appetite for NaCl when confronted with it for the first time. He states that with mammals there is a universal liking for NaCl, and he considers this to be indicative of an inherited behaviour pattern which has conferred survival advantage. This primitive attraction for salt was modified by metabolic conditions, as is evidenced by the increased intake of NaCl solution which occurred when NaCl loss was caused by adrenalectomy.

Previous publications from this laboratory have described the preparation and maintenance of sheep which have a permanent unilateral parotid fistula (Denton, 1956, 1957*a*; Denton, Goding & Wright, 1959), and lose 1–4 l./day of alkaline parotid saliva containing 170–680 m-equiv of $NaHCO_3$. An animal prepared in this manner is an excellent subject for the study of physiological adaptations in the face of a controlled and precisely defined stress on the *milieu intérieur*. This paper reports experiments on the voluntary intake by sheep of Na-containing solutions, and the effect of Na^+ deficiency caused by loss of saliva on this voluntary intake.

METHODS

Twenty sheep of Merino or Merino cross-breeds were used. Six were ewes, the remainder were wethers. They were 1–7 years old. Apart from the normal sheep in Table 1 all had Wright-type permanent unilateral parotid fistulae and ipsilateral carotid artery loops. A number had contralateral carotid artery loops also. Three sheep, T.P. 1, T.P. 4, and T.P. 12, had the left adrenal gland transplanted to a combined carotid-artery–jugular-vein skin loop in the neck (McDonald, Goding & Wright, 1958) and the right adrenal gland removed.

Most of the sheep were purchased from sale yards, and their previous history was unknown in relation to the possibility of access to salt licks or to bore water containing salt. The animals were kept in stainless-steel metabolism cages which effectively separated excreta,

and permitted collection of uncontaminated specimens of urine, saliva and faeces (Denton, 1956). In experiments where electrolyte analyses were made on the saliva each day, the anterior compartment was washed daily with distilled water, and, in the case of external electrolyte balance studies, these washings were analysed also. Sheep were fed daily on 0·8–1 kg of a mixture of equal parts of oaten and lucerne chaff. This food was mixed so as to give large batches of uniform composition and was analysed at intervals. Thus the Na^+ intake from food was known and was approximately 100 m-equiv/day. For external electrolyte balance studies several food analyses were made in the course of the experiment, and the mixing was very thorough.

TABLE 1. Voluntary intake of various electrolyte-containing solutions and water by normal sheep, and sheep with a unilateral parotid fistula

Name of sheep	Experimental conditions	No. of days	Average voluntary intake (l./day)				Total volume (l./day)	Total m-equiv Na^+/day	Saliva volume (l./day)
			Water	NaCl	$NaHCO_3$	KCl			
Dido	Normal	16	0·91	0·51	0·28	0·06	1·76	332	—
	1 hr/day	9	—	0·08	0·04	—	—	50	—
	Normal	5	0·68	0·39	0·45	0·02	1·54	353	—
Lucy	Normal	10	1·40	0·83	0·88	0·65	3·76	718	—
Dolly	Normal	11	0·77	0·71	0·22	0·28	1·98	391	—
Edward	Normal	13	1·55	0·77	0·02	0·03	2·37	323	—
	1 hr/day	8	—	0·18	0	0	—	76	—
	Normal	5	1·24	1·11	0	0	2·35	466	—
Fred	Normal	11	1·81	0·88	0·74	(not offered)	3·43	680	—
	5–10 days post-op.	5	0·24	0·37	1·31	—	1·92	706	—
Fido	Normal	6	0·67	0·10	0·01	0·97	1·75	46	—
	17–25 days post-op.	8	0·95	0·48	0·27	0·99	2·69	305	0·81
Disraeli	Normal	15	1·01	0·51	0·26	0·38	2·16	323	—
	15–20 days post-op.	5	2·19	0·24	0·75	0·32	3·50	416	1·67
Charlie	Normal	8	1·99	0·11	0·01	0·01	2·12	50	—
	1–7 days post-op.	6	2·77	0·05	0·66	0·02	3·50	298	1·86
Fraser	Normal	8	1·71	0·14	0·08	0·08	2·01	92	—
	5–10 days post-op.	5	2·11	0·23	0·55	0·11	3·00	328	2·21
Cassie	Normal	13	0·67	0·42	0·24	0·38	1·71	277	—
	11–14 days post-op.	3	1·53	0·46	0·12	0·58	2·69	244	1·49
	21–27 days post-op.	6	1·25	0·39	0·53	0·62	2·79	386	1·33
	10 months post-op.	4	—	—	—	—	—	443*	2·34

* $NaHCO_3$ only available.

Solutions. Analytical reagent standard chemicals were used to make the solutions of electrolyte offered the animals. The appropriate amount was weighed out, and dissolved in tap water. The solutions were offered to the animal in stainless-steel bins, 30 cm × 20 cm × 15 cm. Each cage had an opening 45 cm long and 30 cm high at the anterior end on each side and two bins containing fluid could be attached to the outside on each side. The front door of the cage had a hinged flap 38 cm wide and 21 cm high and this was raised and the food bin attached in this position. With experiments lasting some weeks when four fluids (H_2O and solutions of NaCl, $NaHCO_3$, and KCl) were offered to the animal each day, the four places on the cage were numbered, and the position of the fluids was varied randomly on the basis of tossing two coins. The same amount of fluid was placed in each tin, and usually the volume was 3–4 l. The evaporation from the tins under summer conditions was approximately 50–100 ml./day. For this reason, solutions which were untouched or partly

drunk, and had been made up to original volume by addition of stock solution were discarded every third day. Specimens were taken from the bins at intervals and analysed to confirm the accuracy of the concentration. In experiments where the animal had access to the solutions for 1 hr only each day evaporation did not have to be taken into account.

Methods of observing behaviour. In some experiments the animals had access to food and various solutions continuously. In others the animal had access to the solutions for 15–60 min only at a standard time on the experimental day. In these circumstances the observer sat about 8 ft. (2·4 m) from the cage and recorded the animal's sampling behaviour. About 15 min before the sampling experiment two large steel sheets were hung on the inner sides of the cage, so that the animal was unable to see or explore the solutions as they were placed in position. At zero time the guards were removed and any of the following acts were recorded as a sampling 'episode'—dipping the head into a tin, dipping the jaw in the solution, taking the solution in the mouth, or drinking the solution. The number of episodes during the period of access was recorded. Under some conditions behaviour could be recorded satisfactorily by a shorthand notation, but under others the rapidity of movement and sampling required that the observer quietly described proceedings into the microphone of a tape recorder. The typewritten record of the episodes was then completed for time sequence by running the original tape record with a stop-watch. In the experiments where rate of drinking was measured, the act was timed with a stop-watch and the container removed, weighed quickly, and returned to its position.

Sometimes it was of interest to determine the preference of a sheep for a particular solution without allowing it to drink enough to alter its state of balance. Two cups, 10 cm in diameter and 5 cm deep were set 2·5 cm apart on a small tray which had a handle 43 cm long. At zero time the front door of the cage was opened and the animal had access to 50 ml. of solution in each cup. In the appropriate circumstances the animal would smell or taste one solution and reject it whereas it avidly drank the other and licked the cup.

Saliva secretion rate was measured over short intervals by counting the drops of secretion as they fell from the dependent point of the Wright fistula. The sheep, P.F. 33, P.F. 48, Ned and T.P. 12, which were used for experiments involving detailed observation of sampling behaviour, had all been in the laboratory for 1–3 years and were thoroughly accustomed to having members of the laboratory in their immediate vicinity for many hours each day. They were confident and apparently quite undisturbed by any procedure involved in these experiments.

Chemical methods were those described by Denton (1956).

RESULTS

The voluntary intake of electrolyte containing fluids by normal sheep

This was observed in ten sheep. They were offered four fluids (water, NaCl and $NaHCO_3$ (420 m-equiv/l.) and KCl (130 m-equiv/l.)). The solutions were available all day, the position being varied randomly each day. The sheep were given their fixed ration at the same time each day, providing a Na intake of 50–100 m-equiv/day. Usually the animals were offered water in all four tins for 5–10 days before the observation period began.

Table 1 shows the voluntary intake of the ten normal sheep. All the animals drank some Na^+-containing fluid, and with one exception (Lucy) there was a preference for NaCl over $NaHCO_3$. The average daily Na intake in different sheep varied from 46 to 718 m-equiv. Sometimes, but not

invariably (Figs. 1 and 2), the main intake was in the first days of the period, the amount being reduced after initial drinking episodes on the first 1 or 2 days. Urinary analyses were made in some animals and showed a substantial Na excretion. In the case of Lucy Na excretion exceeded 1000 m-equiv on some days. With the exception of Fido and Lucy, little KCl solution was drunk. In two sheep, after the control period, the electrolyte solutions were made available to the sheep for 1 hr/day only. The intake observed was small.

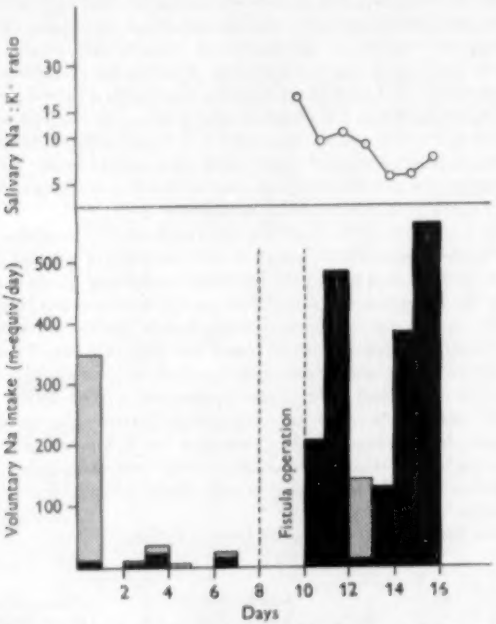

Fig. 1. Charlie, Merino wether. The voluntary daily intake of solutions of $NaHCO_3$ (■ 420 m-equiv/l.) and NaCl (▨ 420 m-equiv/l.) before and after the establishment of a permanent unilateral parotid fistula. The $Na^+:K^+$ ratio of the parotid saliva is shown also. KCl (140 m-equiv/l.) was offered concurrently but there was little or no intake (Table 1). These solutions and water were available all day.

With Charlie (Fig. 1), the behaviour before operation was examined in some detail. After an intake of NaCl when the solutions were offered first, the animal showed little interest in the electrolyte fluids. On one occasion during this pre-operative period the animal had no access to any fluid for 48 hr, and it was obviously thirsty at the end of the period. It was offered various solutions on the 'preference tester' (see Methods). On the first trial, $NaHCO_3$ and H_2O were offered. It encountered $NaHCO_3$, tasted it

and passed quickly to the other tray where it found water which it drank. Eleven tests were made and it drank H_2O whenever this was included but rejected NaCl, $NaHCO_3$ and KCl. Despite variation of position of various solutions offered, it appeared that after initial testing the sheep could identify the solutions on smell alone in the subsequent tests, and would, after moving its head across the tray, proceed immediately to water and drink. This animal began to drink $NaHCO_3$ on the first day after a parotid fistula operation, having shown no interest in it during the pre-operative period.

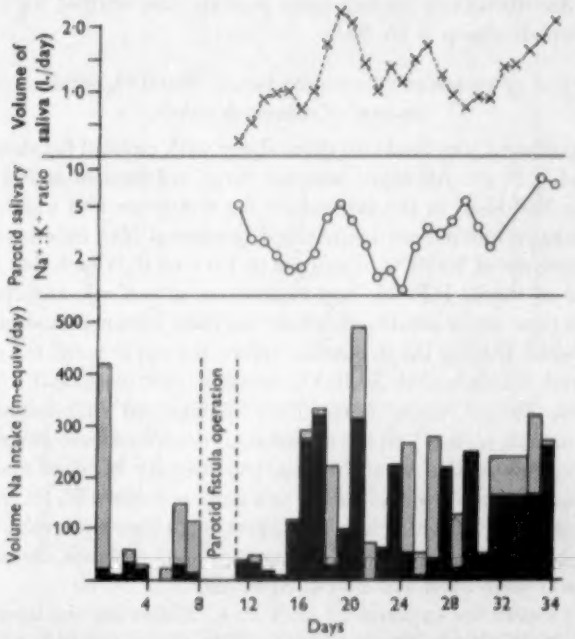

Fig. 2. Fraser, Merino wether. The voluntary daily intake of solutions of NaCl and $NaHCO_3$ before and after establishment of a permanent unilateral parotid fistula. The volume of saliva secreted each day, and the parotid salivary Na^+:K^+ ratio are shown also. The conditions and symbols similar to those for Fig. 1.

The voluntary intake by sheep with parotid fistula

In six sheep of Table 1 a parotid fistula operation was carried out, and the effect of saliva loss on the voluntary intake of the solutions was compared with the initial period. Figures 1 and 2 show the effect of the parotid fistula on the daily intake of two of the sheep. In no case was the daily saliva loss large (i.e. more than 2 l. = a Na^+ loss of *ca.* 300 m-equiv/

day). Where the pre-operative Na intake was small, there was a significant post-operative increase. In four of the six animals there was a clear post-operative preference for $NaHCO_3$ over NaCl—a more appropriate choice in the face of the $NaHCO_3$ loss from the fistula. With the two which did not show the preference the volume of fistula loss was small. Further observations recorded in sections below confirm a clear-cut preference for $NaHCO_3$ by sheep which lost a large volume (3–4·5 l. daily) from a parotid fistula. The salivary Na^+:K^+ ratio analyses in Figs. 1 and 2 show that the animal's voluntary intake was inadequate to prevent some degree of Na^+ deficiency during some periods; the normal Na^+:K^+ ratio of a Na^+-replete sheep is 20–30.

The effect of variation of concentration of $NaHCO_3$ solution on the amount of voluntary intake

This experiment was made on three sheep with parotid fistulae (P.F. 33, T.P. 1, and T.P. 4). All three secreted large volumes of saliva (3–4·5 l./day). They had been in the laboratory for 2–4 years and until 2 months before this experiment were maintained in normal Na^+ balance by giving 600–830 m-equiv of $NaHCO_3$ dissolved in 1·5 l. of H_2O each day by rumen tube. One of them, P.F. 33, had experience of self-selection procedures during this time under conditions where the daily intrarumenal supplement was withheld. During the 2 months before the experiment the sheep had been offered 2 l./day of a $NaHCO_3$ solution (300 m-equiv/l.) instead of being given Na by rumen tube. They maintained themselves in good condition on this régime. In the experiment described here the sheep were given 0·8 kg/day of the usual diet (*ca.* 100 m-equiv Na/day) and had free access to water. They also had access to a large volume of $NaHCO_3$ solution, the concentration of which was varied at 3–10 day intervals. Allowing the Na^+ content of food, the net negative Na^+ balance caused by the fistula would have been 400–580 m-equiv/day.

Figure 3 shows the experiment on T.P. 4. Following the long period of access to $NaHCO_3$ at 300 m-equiv/l., T.P. 4 was offered 950 m-equiv $NaHCO_3$/l. for 2 days. It drank 0·7 and 0·44 l., i.e. 665 and 420 m-equiv Na. On the third day the concentration was changed to 119 m-equiv/l. and the animal drank 4·86 l. = 583 m-equiv of Na on this day. The average intake for the period of 10 days with this concentration was 535 m-equiv/l. and the adaptation to this large change of concentration was made during the first day it was offered. During the next 10 days the concentration was 238 m-equiv/l. and the average Na intake was 616 m-equiv. In this instance the adaptation to change was slower, the average intake for the first 3 days being 794 m-equiv/day. At 476 m-equiv $NaHCO_3$/l. the average daily intake of Na was 428 m-equiv, and when the

concentration was increased again to 952 m-equiv/l. the average daily Na intake was 350 m-equiv. In both instances the animal adjusted the volume drunk to the concentration on the first day of change. After a further period of 7 days with concentration at 476 m-equiv $NaHCO_3$/l., 476 m-equiv of $NaHCO_3$ was added each day to the sheep's food. As Fig. 3 shows, it drank practically no $NaHCO_3$ solution after the first day.

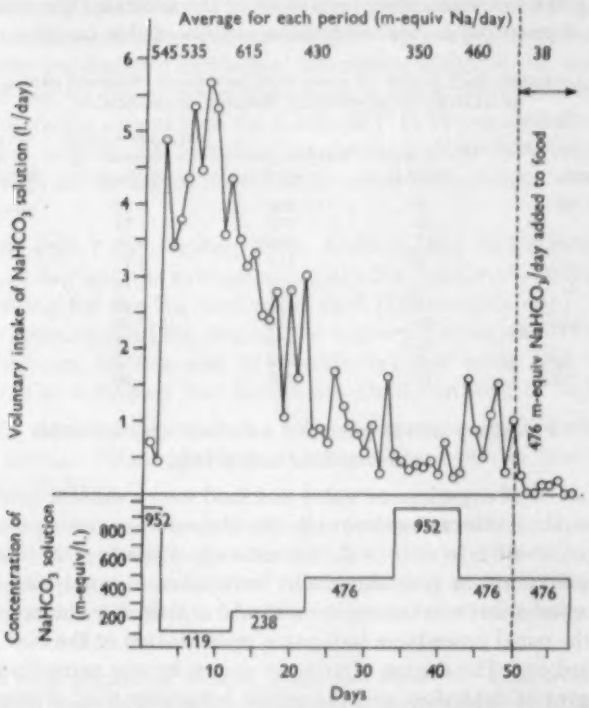

Fig. 3. T.P. 4, Merino wether. The volume drunk each day of a solution of $NaHCO_3$, the concentration of which was varied as shown in the lower section of the figure. Water was available continuously. The average intake (m-equiv Na/day) for each period is shown also at the top of the figure. After 50 days, 476 m-equiv of $NaHCO_3$/day was added to the food.

Table 2 summarizes the experiments on two other sheep. P.F. 33 was able to adjust intake to change of concentration but the adaptation was not made always on the first day of change of concentration. With T.P. 1, in two periods the intake exceeded considerably the daily loss of Na^+ from the fistula.

Summarizing, this series of experiments showed that the animals drank

voluntarily an amount of $NaHCO_3$ solution which approximated to the loss each day from the fistula. They were able to do this in the face of variation in concentration of solution offered and, in many instances, they adapted to a change of concentration immediately (i.e. during the first day the change was made). It appeared as if the sheep were able to 'multiply volume by concentration', which provoked interesting questions concerning the exact sampling behaviour of the animals. The next section and a subsequent paper deal with some aspects of this question.

TABLE 2. Voluntary daily intake, by sheep with permanent unilateral parotid fistulae, of $NaHCO_3$ when offered in different concentrations

Sheep	No. of days offered	$NaHCO_3$ concn. (m-equiv/l.)	Average volume drunk (l./day)	Average Na intake (m-equiv/day)
P.F. 33	9	238	2·13	507
	11	476	1·21	576
	11	952	0·70	666
	9	119	4·77	568
T.P. 1	5	952	1·13	1076
	7	119	4·46	531
	10	238	1·95	464
	12	476	0·77	366
	9	952	0·52	495
	17	476	1·96	932

Voluntary intake when Na solutions were available for one hour a day only

In this series of experiments water and food were available continuously to the animal. At the same time each day the solutions were presented for a period of 15–60 min only and, immediately following the test period, saliva and urine of the preceding 24 hr were taken for analysis. A feature of these experiments was the excitement and anticipatory behaviour of the sheep as the usual procedures indicating presentation of the Na^+ solutions were carried out. The degree of urgency shown by the animal was related to the degree of depletion, and the motor behaviour (foot stamping, etc.) was associated with a variety of visceral responses including salivary conditioned reflexes (Denton *et al.* 1960). During the period of access the number of episodes of sampling solutions, and the exact sequence of smelling and tasting them were recorded.

Experiments were made on P.F. 33, P.F. 40 and P.F. 43. Four randomly placed solutions (water, NaCl and $NaHCO_3$ (420 m-equiv/l.) and KCl (134 m-equiv/l.)) were available to the sheep for 1 hr only. Intake was observed during control periods when the sheep received 595–830 m-equiv/day of $NaHCO_3$ by intrarumenal tube and during Na^+ deficiency produced by withdrawal of this supplement. With P.F. 33 the voluntary intake during Na^+-replete stage was negligible (4 m-equiv Na during the

hour each day) and during the 7 days of depletion averaged 298 m-equiv of Na during the hour each day with a clear preference for $NaHCO_3$. The average number of sampling episodes involving the Na containers was 5 in the hour during the control period, whereas during deficiency there were 30–40 episodes in the hour. However 75% of the voluntary intake occurred in the first 5 min. Following readministration of the Na supplement, voluntary intake and interest in the solutions declined precipitately and the animal sometimes did not sample any solutions until 8–27 min after the observation hour had begun. The results with P.F. 40, and P.F. 43 which had had no experience of self-selection procedure before the experiment, were similar except that the intake of P.F. 43 was entirely as NaCl.

A further experiment including an external electrolyte balance was made on P.F. 33, lasting 34 days during which the solutions were offered in four ways:

(1) For the first 7 days water, NaCl, $NaHCO_3$ and KCl solutions were available all day and the average voluntary Na^+ intake was 602 m-equiv/day. Allowing for the Na content of food (116 m-equiv/day), the total Na intake approximated Na output. The salivary Na loss was 676 m-equiv/day, the urinary Na loss was 22 m-equiv/day and faecal loss was 3 m-equiv/day. The voluntary Na^+ intake was almost entirely of $NaHCO_3$.

(2) For the next 7 days, when the solutions were available each day for 1 hr, the average voluntary Na intake was 560 m-equiv in that hour. It was mainly $NaHCO_3$.

(3) In the next 8 days, when the solutions were available for 1 hr only on every second day, the average voluntary intake was 878 m-equiv in that hour. Approximately one third of this intake was as NaCl. The average amount by which Na loss in the 2 days exceeded food intake was 930 m-equiv.

(4) In the final period of 6 days, when the solutions were available for 1 hr on every 3rd day, average intake was 1137 m-equiv in that hour. Equal amounts of NaCl and $NaHCO_3$ were drunk. The average amount by which Na loss had exceeded intake by this third day was 1070 m-equiv.

Voluntary Na^+ intake in relation to Na^+ balance when concentration of Na^+ solution was varied

Figure 4 shows another experiment on P.F. 33 under different conditions. Water was available continuously throughout the experiment. The animal was fed at the same time each day. Saliva and urine were collected immediately following the test period when $NaHCO_3$ solution was offered for 1 hr each day, but the concentration of the offered solution was different in periods *b*, *c*, *d*, *e* of Fig. 4.

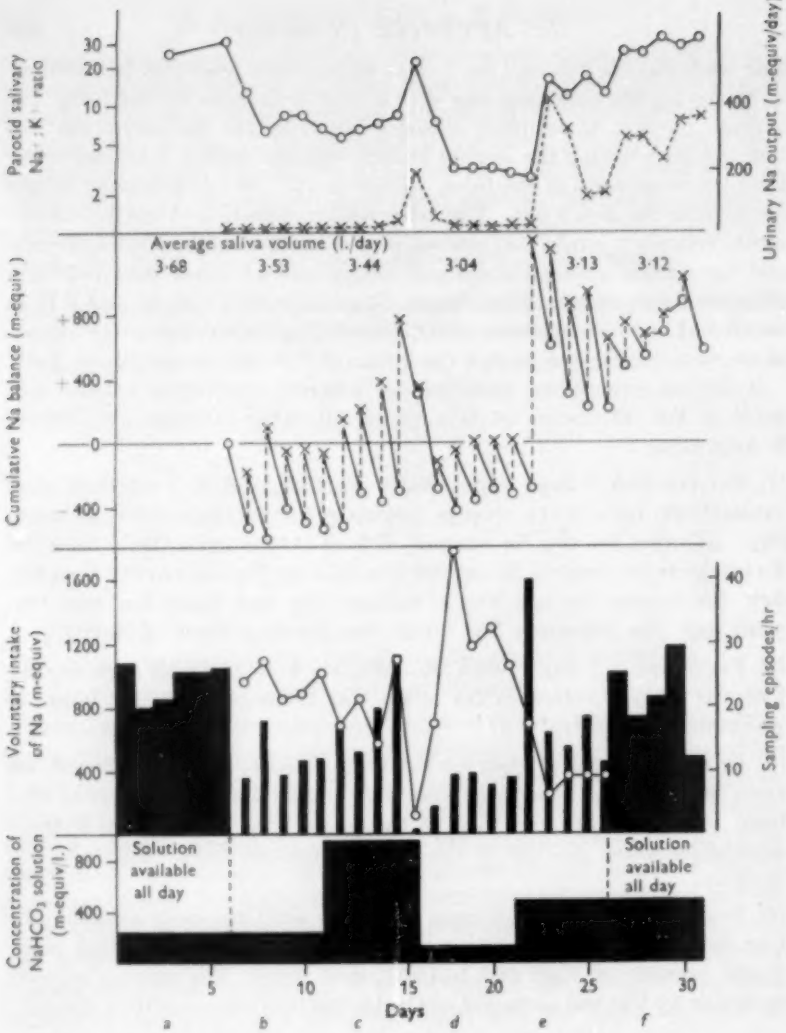

Fig. 4. P.F. 33, Merino cross-bred ewe. The top section records the parotid salivary $Na^+:K^+$ ratio (○—○) and the urinary Na excretion per day (×- - -×). The second section records the cumulative Na^+ balance. The solid line (×—○) records the change of balance (m-equiv Na/day) and the interrupted line (○- - -×) records the change in balance produced by voluntary intake during the hour when access to $NaHCO_3$ was permitted. The average salivary volume per day for each period of the experiment is shown also. The third section shows the voluntary intake of Na^+ when the animal had access all day, and (thin column) when there was access for 1 hr only. The number of sampling episodes during the hour of access (○—○) is shown also. The bottom section records the concentration of the $NaHCO_3$ solution which was offered. Water was available continuously.

For some months before the experiment the sheep had been maintained on a régime involving access to 2 l./day of a solution of $NaHCO_3$ 300 m-equiv/l. It usually drank the lot each day. During the 6-day control period (*a* in Fig. 4) the average intake of the solution continually offered, containing 238 m-equiv/l., was 970 m-equiv Na/day. The average saliva volume, 3·68 l./day, involved a daily loss of 680 m-equiv of Na. Hence the voluntary daily Na intake, which varied little, was considerably greater than fistula loss. Consistent with this, the salivary Na^+:K^+ ratio was normal. No urinary analyses were made, but as is indicated by the final section (*f* of Fig. 4) the excretion was probably 200–400 m-equiv/day.

During the period *b* of Fig. 4 the sheep had access to 238 m-equiv $NaHCO_3$/l. for 1 hr each day. The balance study chart is constructed with an assumption that, at the end of the period of continuous access to $NaHCO_3$, the animal was in Na^+ equilibrium. The solid line (×—○) indicates the change in external Na^+ balance during the 23 hr preceding the 1 hr test, and shows a decrease of Na content of the animal because the fistula loss greatly exceeded Na intake in food. The interrupted line (○- - -×) shows the change in Na^+ balance produced by the voluntary intake of Na during the hour period of access. The number of episodes of sampling the $NaHCO_3$ solution and water during the hour are shown on the voluntary Na intake section of the figure. The average intake in the hour each day of the 238 m-equiv $NaHCO_3$/l. was 476 m-equiv. As the cumulative Na^+ balance shows, the 23 hr negative Na^+ balance was usually over 500 m-equiv, so that the voluntary intake was about adequate to restore Na^+ equilibrium. Consistent with the fact that the external Na^+ balance was negative during most of the 23 hr period, the Na^+:K^+ ratio in the 24 hr salivary collections was about 7 and the excretion of Na in the urine was negligible.

When, in section *c* of Fig. 4, the concentration of $NaHCO_3$ solution offered for 1 hr each day was changed to 952 m-equiv/l., the voluntary intake increased and the external balance became positive for a significant period of each day. On the fourth day the animal drank a large amount and the Na^+:K^+ ratio of the next 24 hr specimen of saliva was normal and the kidneys excreted 181 m-equiv of Na. On the fifth day the sheep drank very little Na solution, and the record of sampling episodes reflected this lack of interest in the presence of a positive Na^+ balance. The average voluntary intake for the period was 624 m-equiv Na/day.

In period *d* of Fig. 4 the concentration of $NaHCO_3$ solution was reduced to 119 m-equiv/l. On the first day the number of sampling episodes increased but the intake was small. On the second day the number of sampling episodes was the largest for the experiment and the amount drunk was almost adequate to return the Na^+ balance to equilibrium. This

involved drinking over 3 l. of the Na solution within the hour. During the remainder of the period the voluntary intake approximated the amount of Na^+ deficiency and thus was just adequate to return the external balance to equilibrium at the beginning of each day. The over-all state of Na^+ deficiency prevailing was reflected in the low salivary Na^+:K^+ ratio (approx. 3·0) and the complete renal conservation of Na. The fact that the animal was maintaining a Na^+ equilibrium involving a considerable residual deficiency is reflected also in the decreased Na^+ loss during the 23 hr. The reduced salivary Na^+:K^+ ratio caused the Na^+ loss to be 200–300 m-equiv/day less than in section *c* when the animal was drinking the 952 m-equiv $NaHCO_3$/l. solution. The average voluntary intake for the period was 313 m-equiv Na/day. The increased number of sampling episodes during this period of 119 m-equiv $NaHCO_3$/l. was associated with some clear-cut changes in the animal's behaviour, which will be described in detail in a subsequent paper dealing with conditioned salivary reflexes associated with Na appetite.

In period *e* of Fig. 4, on the first day the animal had access to the $NaHCO_3$ solution at 476 m-equiv/l. concentration, it drank immediately 3·28 l. = 1561 m-equiv Na. It sampled the solutions much less on the following day but in an initial episode it drank 1·26 l. = 600 m-equiv of Na. The very large Na^+ intake, greatly in excess of deficit, resulted in a large positive Na^+ balance. The salivary Na^+:K^+ ratio returned to normal and there was a large urinary Na excretion which persisted throughout the period. During the period the balance was persistently positive, though it approached Na^+ equilibrium after 23 hr of salivary loss. No measurements were made which might indicate whether the excess Na^+ in the body was as an increased amount of intrarumenal Na, an increased extracellular Na or as storage in a depot such as bone. The average number (10) of sampling episodes for the period was considerably less than the preceding period. The average voluntary intake for the period was 784 m-equiv Na/day.

Finally, in period *f*, the animal was permitted access to the 476 m-equiv $NaHCO_3$/l. during the whole day, and the intake was recorded also during the usual 1 hr period. A small amount was drunk during the hour, the over-all intake for the period (848 m-equiv Na/day) approximating that during the preliminary control period (*a*), when the solution was 238 m-equiv $NaHCO_3$/l. The animal remained in positive Na^+ balance, the salivary Na^+:K^+ ratio was normal, and there was a large urinary Na excretion.

It is convenient here to record one further aspect of the drinking behaviour in the experiment of Fig. 4. Figure 5 shows the percentage of the intake drunk during each 5 min period of the hour during which each

concentration was offered. The principal fact emerging is that with the 952 m-equiv/l. concentration the animal took 90% of the total within 5 min, and virtually all within 15 min. From 15 min onwards it drank considerable amounts of water. The same pattern held with 476 m-equiv $NaHCO_3$/l. except that no water was taken during the hour. By contrast,

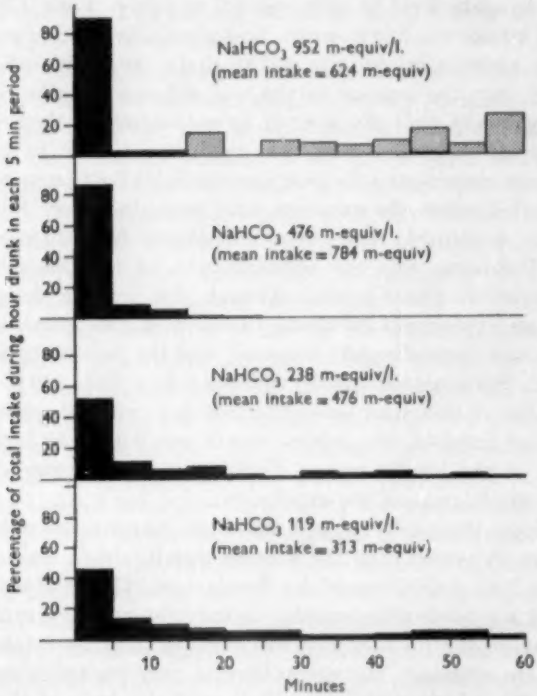

Fig. 5. P.F. 33, Merino cross-bred ewe. The percentage of the voluntary intake drunk in each 5 min period of the hour of access at the various concentrations at which the $NaHCO_3$ solution as offered (▨ H_2O, ■ $NaHCO_3$). The figure shows also that only with the 952 m-equiv/l. solution was there any voluntary intake of water during the hour. The average of the 5 days in each of periods *b*, *c*, *d*, *e* of Fig. 4 is shown.

with the 119 m-equiv/l. concentration only 44% of the intake was taken in the first 5 min, and drinking continued over the whole hour. This was seen also with the 238 m-equiv $NaHCO_3$/l. and in neither case was any water drunk during the hour.

A study on Ned similar to Fig. 4 extended over 67 days divided into periods of approximately 7 days. With 238 m-equiv $NaHCO_3$/l. available all day, the voluntary intake was 239 m-equiv/day. When the same

solution was available for 1 hr/day only, the average intake was 243 m-equiv. With 952 m-equiv/l. the average intake in the hour was 362 m-equiv, and with 119 m-equiv/l. the average intake was 100 m-equiv. After a 7-day period with the 119 m-equiv $NaHCO_3$/l. available all day (intake equalling 279 m-equiv/day) average intake by the animal when the solution was available again for 1 hr only was 223 m-equiv. With 476 m-equiv/l. the average intake was 329 m-equiv, and when this solution was available all day the average intake was 351 m-equiv; and with 952 m-equiv/l. available all day, the average intake was 426 m-equiv/day. The pattern of voluntary intake over the hour of access resembled that recorded for P.F. 33 (Fig. 5). A detailed study was conducted on T.P. 12 during which the three usual electrolyte solutions were available for 15 min only. During a control period, when the solutions were presented every third day, the observations confirmed those reported above for 420 m-equiv Na/l. solutions. Following this the concentration of the Na solutions was varied from 220 to 880 m-equiv/l. Though this was the sheep's first experience of an experiment involving variation of concentration, the total intake remained approximately constant, and the preference for $NaHCO_3$ was retained. For example, one day it drank 2·61 l. of the 220 m-equiv Na/l. solutions in the 15 min, whereas on the next day, when the concentrations were increased fourfold, the volume drunk was 0·56 l. In both instances about 75% of the intake was as $NaHCO_3$ and the sheep rejected KCl throughout the 32 days of the experiment.

Summarizing, the results showed that when Na solutions were presented for a very short period only the animals usually drank enough to repair most of the Na^+ deficit caused by fistula loss. Under these conditions they showed a considerable capacity to vary the volume drunk when the concentration of the Na solutions was changed, so that intake remained approximately constant. The sheep became very excited when presentation of the Na solutions was imminent, and the bulk of the voluntary intake occurred during the first 5–10 min of access, following which there was a decline of interest in the solutions.

DISCUSSION

In the experiments where normal sheep were offered the salt solutions to drink some animals had a large voluntary intake though there was clearly no deficiency of Na^+. Generally, there was a preference for NaCl over $NaHCO_3$. The concentration of the Na solutions offered was 420 m-equiv/l. and in some instances the amount of water drunk was such that the Na^+ concentration of the daily fluid intake was greater than that of blood. Thus the voluntary Na^+ intake resulted in a very large urinary

excretion of Na at concentrations as high as 380 m-equiv/l. There was considerable variation within the small group of animals studied. The results were consistent with Richter's (1956) findings of liking for salt in the absence of metabolic need. However, unambiguous evidence that this behaviour in sheep was not due to previous experience depends on the outcome of experiments with sheep whose previous history in relation to salt access is known with certainty.

The effect of making a parotid fistula which caused loss of Na^+ was clear-cut. In the sheep which had a small voluntary Na intake beforehand there was a large increase, and usually this involved a preference for $NaHCO_3$ over NaCl. In the instance of the sheep which had a large Na intake before operation there was usually an increase in the proportion of $NaHCO_3$ drunk. There was one exception (P.F. 43) which after 4 days of Na depletion drank 3·46 l. of NaCl, and in the following month always drank NaCl and rarely drank much $NaHCO_3$ though it often tasted it. All sheep with long experience of self-selection, e.g. P.F. 33, P.F. 40, P.F. 48 and T.P. 12, showed a preference for $NaHCO_3$, although they frequently drank a significant amount of NaCl every few days. This preference was less apparent when the animal had been Na-depleted for 2–4 days and showed a sense of urgency and was very excited by the impending presentation of the Na solutions. The selection of a predominance of $NaHCO_3$ over NaCl was a more appropriate choice in the face of the biochemical distortion produced by the predominantly $NaHCO_3$ loss from the parotid fistula. The mechanism of selection has not been investigated. One possibility is that the fall in pH and HCO_3 concentration of the arterial blood perfusing the taste buds is in some way responsible, an hypothesis it should be possible to test by making appropriate infusions into the carotid artery loop supplying a 'vascularly isolated' tongue during the act of self-selection (Denton *et al.* 1959). An alternative possibility is that in the course of random trial and error drinking of the solutions, the animal took enough $NaHCO_3$ to produce subsequently a particular sense of alleviation of need, and that retroactive association of benefit and taste established a preference for that solution.

The quantitative study of voluntary intake in relation to degree of Na^+ deficiency showed that, when the solutions were available all day, many animals maintained themselves in normal balance with a normal salivary Na^+:K^+ ratio. Others maintained an oscillation of balance involving some residual Na^+ deficiency as shown by a salivary Na^+:K^+ ratio of 3–10, and little or no urinary Na excretion. A striking finding was the ability of the sheep to adjust Na intake to need by varying the volume of Na solution drunk per day in the face of change of Na^+ concentration of the solution offered.

This self-regulating ability was clearer from studies in which the solutions were available for 15–60 min only each day or on every second or third day. The external balance studies showed that generally the amount of Na solution drunk in the hour was adequate to replace the Na^+ deficit during the previous 23 hr. The study on P.F. 33 (Fig. 4) indicates that judgement of voluntary intake was usually accurate. This returned the Na^+ balance to equilibrium but, as Na^+ loss continued during the next 23 hr, the balance was negative during most of the day, a finding reflected by low salivary Na^+:K^+ ratio and urinary Na^+ conservation. If, because of an episode with very large Na^+ intake, there was a large positive balance, the intake was usually reduced next day. On the other hand, once the positive balance was established, sometimes the animal maintained a status involving fluctuation of balance on the positive side of Na^+ equilibrium, an observation consistent with the primary finding that Na^+-replete non-fistulated sheep will drink the solutions. The findings in this respect were somewhat suggestive that rate of change of Na^+ balance in the preceding period was influential in determining drinking behaviour as well as the apparent primary determinant of the actual state of Na^+ balance. Consideration of the balances and the increased intake when there were 2–3 days of depletion highlights one other fact shown graphically in Fig. 5. As much as 80–90 % of the intake occurred during the first 5 min of access to the solution. The findings are suggestive that the action specific energy (Lorenz, 1950; Tinbergen, 1951; Haldane, 1956) for Na appetite is accurately determined by metabolic balance. The specificity of the appetite for Na solutions was confirmed frequently by the rejection of food, H_2O or KCl by the restless and excited animal which continued to bleat and jump up and down until Na was offered. The specificity of the appetite was also clear from multiple analyses of the rejection and acceptance pattern by the animal when the four fluids were presented. The fact that this action-specific energy was discharged to a large extent in most instances by a single drinking act within the first 5 min of access to fluid raises the fascinating question as to how the relevant information on satiation is computed by a postulated neural centre determining appetite. For example, with T.P. 12 the animal had had no previous experience of variation of Na^+ concentration of the offered fluid until this occurred under the régime of access for 15 min only. Yet on the different days when it met solutions of NaCl and $NaHCO_3$ of 220 and 880 m-equiv/l. for the first time it immediately adjusted the volume drunk so that the total intake was approximately equal at the two differing concentrations. The question whether drinking (presumably a satiating consummatory act (Tinbergen, 1951)) is eventually inhibited as a result of an adequate number of taste bud impulses coupling centrally with an adequate number of oesophageal

'metered' swallowing acts turns on appropriate future experiments on animals with oesophageal fistulae. There seems no possibility that absorption into the blood would be rapid enough to raise the question of this inhibiting the act of drinking (Le Magnen, 1953).

Considering the cause of the Na appetite of deficient sheep, two of the possibilities are that either the increase of aldosterone concentration in blood or the changed composition of saliva in the mouth caused by aldosterone secretion (Denton *et al.* 1959, 1960) stimulate this behaviour. However, normal self-regulatory capacities have been observed in a large number of bilaterally adrenalectomized sheep with parotid fistulae which have been maintained in our laboratory by the self-selection régime. Voluntary ingestion of $NaHCO_3$ adequate to correct deficiency has been seen at times when there was no residual action from electrolyte-active steroid such as DOCA as shown by the secretion of parotid saliva of normal composition despite Na^+ depletion. These observations are evidence against either of the above possibilities as a stimulus of Na appetite. In relation to the spontaneous ingestion of salt solution by sheep which are demonstrably in normal Na^+ balance, a finding which seems to have been confirmed by many field observations, it seems probable that the innate releasing mechanism is the taste of salt encountered during exploratory tasting. The basis of the fairly widespread propensity to ingest salt upon encountering it lies presumably in the selection of an inherited pattern conferring survival advantage on the species. Na^+ deficiency could occur under a variety of natural conditions particularly in ruminant animals (Denton, Wynn, McDonald & Simon, 1951; Denton, Goding, Sabine & Wright, 1958). Notwithstanding the variation of voluntary intake seen in the Na^+-replete animal, Na^+-deficient animals, with very few exceptions, show an active appetite for Na.

If one considers the thirst mechanism, there appears to be evidence that the action-specific energy for drinking determined by osmotic pressure changes (Wolf, 1958) depends upon a fairly precisely defined region of the hypothalamus (Andersson, Jewell & Larsson, 1958) and is reinforced by proprioceptor impulses from the dry mouth and pharynx. There is much less evidence on a physicochemical basis of action-specific energy for Na appetite than there is in the case of thirst. With thirst, Wolf (1958) has shown that raising the osmotic pressure of the blood by 1–2%, a degree of change shown by Verney (1947) to release antidiuretic hormone, causes ingestion of water. We have produced the same effect on sheep by slow intracarotid infusion of 4 M-NaCl or 2·7 M glucose, and Andersson *et al.* (1958) have shown that stimulation of a specific region of the mid hypothalamus by injection of 0·1 ml. of 1·5–2% NaCl causes compulsive drinking. With Na appetite, during Na^+ deficiency it is possible that the

accumulation of action-specific energy is determined directly by Na^+ depletion of the specific cells in the central nervous system controlling this appetite. Alternatively, as with the intimately linked problem of control of aldosterone secretion (Farrell, 1958; Denton *et al.* 1959, 1960; Bartter, Mills, Biglieri & Delea, 1959; Coghlan, Denton, Goding & Wright, 1960), a number of peripheral proprioceptor mechanisms (e.g. vascular stretch receptors) might be postulated as the main receptor mechanisms controlling appetite, or secondarily as despatching impulses modifying the activity of the centre postulated above. In the sheep experienced in this regard it has been demonstrated that voluntary Na^+ intake may closely approximate the degree of deficiency. We have referred already to the proposition that the consummatory act of drinking is inhibited at the appropriate point by proprioceptor impulses discharging the action-specific energy. Future careful studies of fistulated animals on the first occasion that they encounter the Na solutions may reveal whether intake is accurately related to degree of deficiency initially, or whether it is retro-active effect from satiation experience which leads ultimately to precise quantitative adjustment of appetite.

If the receptor system determining Na appetite is within the brain or the circulatory system an outstanding problem remaining is how the consummatory act of Na drinking during 3–4 min discharges the action-specific energy and causes a precipitate decline in motivation long before the ingested material could repair the cellular, circulatory, or blood composition abnormalities which may determine appetite. A similar problem exists, of course, in the capacity of the water-depleted camel to correct a deficiency of 50–100 kg in a single drinking act (Schmidt-Nielsen, 1959), but the phenomenon is more complicated in the Na^+-deficient sheep in view of the ability to adjust volume drunk to the concentration of the $NaHCO_3$ solution presented.

SUMMARY

1. Some sheep when offered solutions of Na salts to drink voluntarily ingested considerable amounts though they were in normal Na^+ balance.

2. The establishment of a permanent unilateral parotid fistula caused loss of 1–4 l. of alkaline saliva/day containing 180–700 m-equiv of Na, mainly as $NaHCO_3$. There was a large increase of appetite for Na. The voluntary intake of many animals was adequate to maintain a normal Na^+ balance whilst others oscillated around a balance involving some degree of residual Na^+ deficiency. Offered a choice between NaCl and $NaHCO_3$ these animals showed a clear preference for $NaHCO_3$.

3. If the fistulated sheep were offered continuous access to $NaHCO_3$ solution and water, and the concentration of the $NaHCO_3$ solution was

varied from 119 to 940 m-equiv/l., the animal varied the volume drunk so that Na intake remained relatively constant and approximated the loss from the fistula.

4. If the Na solutions were available to the fistulated sheep for only 15–60 min each day, the animal usually ingested enough to correct its deficit. 80–90% of intake occurred during the first 5 min. When the concentration of Na solution was increased, the volume drunk was decreased proportionately. If access were permitted only on every 2nd or 3rd day, the amount of Na^+ ingested was increased commensurately.

6. In experiments where solutions were offered for a short period only there was a large increase in frequency with which solutions were sampled during Na^+ deficiency. The sheep rarely ingested any KCl solution when it was included amongst the solutions offered. The preference for $NaHCO_3$ over NaCl was maintained during this régime of short-term access.

7. The experimental results have been discussed in terms of concepts of behaviour advanced by ethologists. The action-specific energy for drinking solutions of Na salts appears in the Na^+-deficient animal to be related to the degree of Na deficit, and the problem arises, as in more simplified form with thirst, how the consummatory act of drinking is inhibited. The hypothesis is advanced that there is a central integration of taste impulses from the tongue signalling concentration with pharyngo-oesophageal proprioceptor impulses signalling volume swallowed. The problem remains as to how the consummatory act discharges the action-specific energy long before the ingested material could repair any physiological consequence of Na^+ deficit either within the *milieu intérieur*, or the intracellular fluid of a neural centre controlling Na intake.

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REFERENCES

Andersson, B., Jewell, P. A. & Larsson, S. (1958). An appraisal of the effects of diencephalic stimulation of conscious animals in terms of normal behaviour. In *Ciba Foundation Symposium on The Neurological Basis of Behaviour*, ed. Wolstenholme, G. E. H. and O'Connor, C. M., pp. 76–85. London: Churchill.

Bartter, F. C., Mills, I. H., Biglieri, E. G. & Delea, C. (1959). Studies on the control and physiologic action of aldosterone. *Recent Progr. Hormone Res.* **15**, 311–344.

Coghlan, J. P., Denton, D. A., Goding, J. R. & Wright, R. D. (1960). The control of aldosterone secretion. *PostGrad. Med. J.* **36**, 76–102.

Denton, D. A. (1956). The effect of Na^+ depletion on the Na^+:K^+ ratio of the parotid saliva of the sheep. *J. Physiol.* **131**, 516–525.

Denton, D. A. (1957*a*). The study of sheep with permanent unilateral parotid fistulae. *Quart. J. exp. Physiol.* **42**, 72–95.

DENTON, D. A., GODING, J. R., SABINE, J. R. & WRIGHT, R. D. (1958). Adaptation of ruminant animals to variation of salt intake. *UNESCO/NS/AZ/382*, Teheran Symposium Paper No. 31, October 1958.

DENTON, D. A., GODING, J. R. & WRIGHT, R. D. (1959). Control of adrenal secretion of electrolyte-active steroids. *Brit. med. J.* ii, 447–456; 522–530.

DENTON, D. A., GODING, J. R. & WRIGHT, R. D. (1960). Control of aldosterone secretion. *Clinical Endocrinology* I, ed. ASTWOOD, E. B. pp. 373–391. New York: Grune and Stratton.

DENTON, D. A., WYNN, V., MCDONALD, I. R. & SIMON, S. (1951). Renal regulation of the extracellular fluid. II. Renal physiology in electrolyte subtraction. *Acta med. scand.* **14**, Suppl. 261, 1–193.

FARRELL, G. (1958). Regulation of aldosterone secretion. *Physiol. Rev.* **38**, 709–728.

HALDANE, J. B. S. (1956). Les aspects physico-chimiques des instincts. In *L'Instinct dans le comportement des animaux et de l'homme*, pp. 545–559. Paris: Masson et cie.

LE MAGNEN, J. (1953). Sur l'inhibition à point de départ gastro-intestinal de la prise d'eau spontanée chez le rat blanc. *C.R. Soc. Biol., Paris*, **147**, 1678–1680.

LORENZ, K. Z. (1950). The comparative method in studying innate behaviour patterns. *Symp. Soc. exp. Biol.* **4**, 221–268.

MCDONALD, I. R., GODING, J. R. & WRIGHT, R. D. (1958). Transplantation of the adrenal gland of the sheep to provide access to its blood supply. *Aust. J. exp. biol. Sci.* **36**, 83–96.

RICHTER, C. P. (1956). Salt appetite of mammals; its dependence on instinct and metabolism. In *L'Instinct dans le comportement des animaux et de l'homme*, pp. 557–632. Paris: Masson et cie.

SCHMIDT-NIELSEN, K. (1959). The physiology of the camel. *Scientific American*, **201**, 140–151.

TINBERGEN, N. (1951). *The Study of Instinct*. Oxford University Press.

VERNEY, E. B. (1947). Antidiuretic hormone and the factors which determine its release. *Proc. Roy. Soc.* B, **135**, 25–105.

WOLF, A. V. (1958). *Thirst*. Springfield, Illinois: Thomas.

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THE ACUTE EFFECT OF PROGESTATIONAL COMPOUNDS ON INTACT RABBIT MYOMETRIUM

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It has been shown by Csapo (1954) that if a strip of rabbit uterine muscle is suspended in an isolated organ bath and is subjected to direct electrical stimulation, then the tension which develops declines immediately when progesterone is added to the bath. This is such a striking effect that attempts were made to demonstrate a similar phenomenon in the intact animal (Schofield, 1955). These attempts were not successful. Recently, however, the effect of progesterone *in vitro* has been confirmed by Stucki & Glenn (1961), who also did further work with related compounds. In the present investigation the problem has been re-examined in rabbits, using an improved technique and additional progesterone-like compounds in order to determine whether, in fact, these substances have any immediate physiological action on myometrial cell function in the intact animal.

METHODS

Immature New Zealand white rabbits weighing 2·5–3 kg were injected daily with 30 μg oestradiol in cottonseed oil for at least 7 days. Experiments were then carried out in the intact animal under pentobarbitone anaesthesia, using the technique already described (Schofield, 1960), and recording isometrically from a horizontal uterine segment which could be subjected to direct electrical stimulation. A fine polythene tube was inserted into the uterine lumen and tied in position along with the electrodes (Fig. 1). In this way injections could be made directly into the cavity of the segment without interfering with the recording or in any way changing the external environment of the uterus. The segment, about 2·5 cm in length, was tied on to the electrodes firmly, but not too tightly, so as to isolate the cavity of the segment without interfering with the blood and nerve supplies. To complete the preparation a Perspex chamber was lowered over the segment, as indicated in Fig. 1, and the abdominal wall was sutured above the flange. A heating coil in the roof of the chamber kept the temperature at 38° C, and moist filter paper below the coil preserved the humidity. The results to be described were observed in a series of 34 rabbits.

Stimuli of 10 V (60 c/s a.c.) and 5 sec duration were applied at 1 min intervals and the responses were recorded on a kymograph; the segment was set at resting length, as in previous experiments (Schofield, 1957). Sometimes the stimulation was continued throughout the experiment, at other times it was switched off to allow the development of spontaneous activity. In the latter case the sensitivity of the myometrium to oxytocin was

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assessed by injecting oxytocin directly into the uterine cavity and by finding the threshold dose injected intravenously.

The test compounds (Table 1) were injected into the uterine cavity through the polythene tube. The volume of each injection was 0·1 ml. but as many as ten consecutive injections of the Krebs's solution were given, and sometimes this total volume of 1 ml. was injected continuously during 15–20 min. The effect of these compounds was determined on the

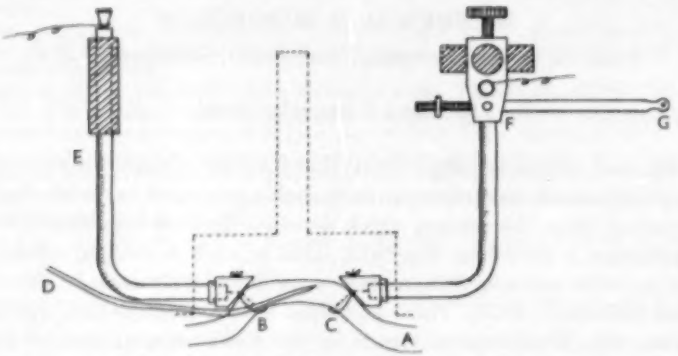

Fig. 1. Diagram of uterine segment (A) tied on to electrodes B and C, with a polythene tube (D) inserted into the lumen for drug injection. The electrode arm (E) is rigid, but the other is movable about a fulcrum (F) and is attached by thread at G to the short arm of an isometric tension lever. The position of the Perspex chamber is indicated in dotted lines.

Table 1. Compounds injected into the uterine segment

Compound	Dosage			
	Vehicle	In 0·1 ml. cottonseed oil	In 0·1 ml. suspension*	In 0·1 ml. Krebs's solution
Progesterone	—	2 mg	2 mg	3·75 μg†
6α-methyl-17α-hydroxyprogesterone acetate (6-MAP, Upjohn)	—	0·4 mg	2 mg	3·75 μg†
Pregnane-3α-20α-diol	—	—	2 mg	—
Pregnanediol glucuronide	—	—	—	0·75 mg
Progesterone enamine HCl	—	—	—	4 mg

* The suspension fluid consisted of (mg): carboxymethylcellulose, 5; polysorbate 80 (polyoxyethylene (20) sorbitan mono-oleate), 4; sodium chloride, 9; benzyl alcohol, 9.

† For these solutions the steroids were dissolved in 1 part dimethylacetamide and then diluted with 800 parts Krebs's solution.

following myometrial properties: (*a*) the tension developed in response to electrical stimulation; (*b*) the spontaneous activity; (*c*) the threshold dose of intravenous oxytocin; and (*d*) the response of the myometrium to intra-uterine oxytocin.

In a few experiments the effect was studied of injecting Na mono-iodoacetate into the uterine cavity. In some experiments Indian ink was injected into the cavity and this showed that there was little or no leakage of the injected fluid from the segment.

Experiments were carried out with progesterone 4-^{14}C to ascertain the degree of absorption from the uterine lumen and the concentrations attained in the blood and uterine wall. An

injection of 2 mg progesterone + 2 μc/4-^{14}C progesterone, either as a suspension or dissolved in oil, was made into the segment and recording was continued for 30 min. The injected segment was carefully removed and the lumen was washed through with 0·9% NaCl solution or with oil and the animal was exsanguinated. The amount of progesterone 4-^{14}C present was then determined in the blood, in the lumen washings and in the uterine segment, which had been separated into myometrium and endometrium, in the following manner: The tissue was extracted four times with 100 ml. portions of 20% (v/v) ethyl acetate in chloroform. Extracts were dried and the dry residues were dissolved in 20 ml. liquid scintillation-counting solvent (2,5 diphenyloxazole, 8 g; 1·4-bis-2-(5 phenyloxazolyl)-benzene, 200 mg; A.R. toluene, 1360 ml.; absolute ethanol, 640 ml.) and appropriate equal aliquot samples were transferred to counting vials. Samples were counted in duplicate at least twice for a duration of 10 min in a Packard Liquid Scintillation Spectrometer. Analyses of unused samples of the material originally injected into the uterine lumen were also made.

In two experiments progesterone 4-^{14}C was similarly injected and thereafter a sample of blood was taken from the jugular vein every 10 min for 1 hr. The samples were then assayed as above. In two other experiments a uterine segment was ligated and injected as above, without the recording apparatus in position. After 30 min the isotope content was estimated in the injected segment, in a distant uterine segment, and in a piece of caecum.

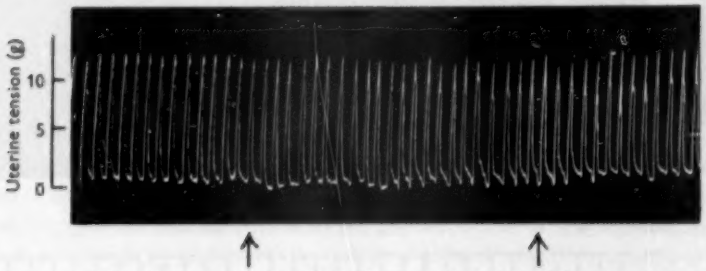

Fig. 2. Rabbit uterus *in vivo*, longitudinal record. Response of the myometrium to electrical stimulation of 10 V and 5 sec duration applied at 1 min intervals. An injection of 2 mg progesterone in 0·1 ml. suspension made into the uterine segment at each arrow.

RESULTS

Progesterone-like compounds injected into the uterine cavity of rabbits did not affect either spontaneous activity or the tension developed in response to direct electrical stimulation (Fig. 2), neither of which showed any change during the subsequent 1–2 hr. The threshold dose of oxytocin injected intravenously in these rabbits was usually 5–10 m-u. and was not affected by the intra-uterine administration of any of the progesterone-like compounds. When oxytocin was injected into the lumen of the segment it produced a definite phase of increased activity (Fig. 3) but the dose required was at least ten times the intravenous threshold dose. Nevertheless, the results showed that oxytocin was absorbed from the lumen of the uterus and, furthermore, that this response was also unaffected by preceding administration of progesterone-like compounds.

Absorption of progesterone 4-^{14}C from the uterine lumen

In two experiments the progesterone 4-^{14}C content of the blood was assayed after injecting into the uterine cavity a solution whose activity was 1,300,000 counts/min. The blood samples, taken every 10 min. showed, in the first experiment, counts varying from 5–21 counts/min/ml. blood, and, in the second experiment, 19–66 counts/min/ml. blood.

The results of the three experiments in which the progesterone content

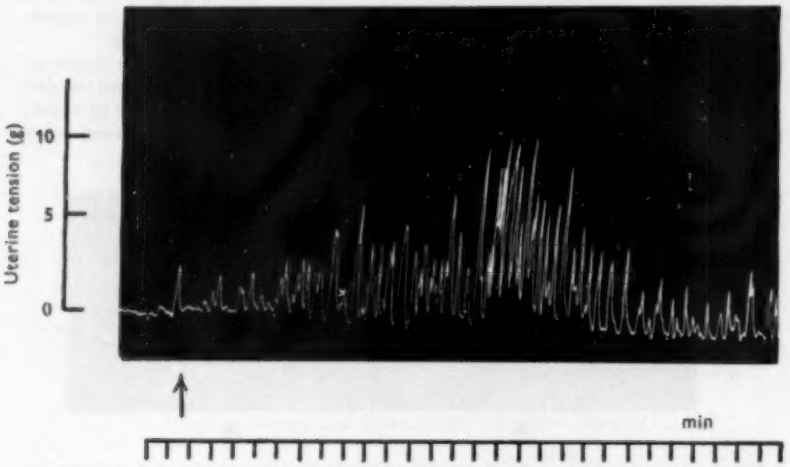

Fig. 3. Rabbit uterus *in vivo*. Effectiveness of intra-uterine injection of 100 m-u. oxytocin in 0·1 ml. 0·9 % NaCl solutions.

TABLE 2. Distribution of progesterone 4-^{14}C 0·5 hr after its injection into the uterine cavity during recording of myometrial activity

Material	Progesterone medium	Total counts/min	Amount of tissue
0·1 ml. progesterone 4-^{14}C material, as used for injection	Suspension	675,758	—
	Oil	637,737	—
	solution	676,547	—
Blood	Suspension	246	80 ml.
	Oil	250	60 ml.
	solution	482	66 ml.
Uterine lumen washings	Suspension	606,607	—
	Oil	201,595	—
	solution	213,200	
Myometrium	Suspension	8,728	—
	Oil	3,135	345 mg
	solution	1,276	381 mg
Endometrium	Suspension	4,153	—
	Oil	2,542	400 mg
	solution	770	322 mg

of uterine tissues was estimated are shown in Table 2. It is evident that the progesterone was reaching the myometrium, though in small amounts, and that in all three experiments the concentration was greater in the myometrium than in the endometrium. Analysis of distant uterine segments and of the caecum showed that the progesterone content was 5% (or less) of that found in the injected segment.

Ineffectiveness of Na mono-iodoacetate. Intra-uterine injections of 0·1 ml. of 0·1 or 0·2 M Na mono-iodoacetate did not produce any significant change in spontaneous or stimulated activity. This is in contrast with the 0·0001 M concentration which, in an organ bath, is effective in abolishing the tension developed by an isolated uterine segment in response to electrical stimulation (Csapo, 1955).

DISCUSSION

In trying to reproduce the acute *in vitro* effects of progesterone in the intact animal the first problem was to get the progesterone to the myometrium in sufficiently high concentrations. When the isolated segment is bathed by progesterone compounds, it is possible to achieve concentrations as high as 10 mg/ml. (Stucki & Glenn, 1961). In the intact animal it is extremely difficult to subject the myometrium to such high concentrations owing to the low solubility of most of these compounds. The method described appeared to be the most efficient available, and, indeed, the results with progesterone 4-^{14}C indicate that after intra-uterine injection of progesterone its concentration in the myometrium of the injected segment is considerably higher than that in a distant uterine segment or in other abdominal organs, e.g. the caecum.

Progesterone, 6-MAP and pregnanediol are almost insoluble in water and therefore only low concentrations of them could be injected in Krebs's solution, but much larger amounts could be injected when in suspension or dissolved in oil. It should be pointed out that Stucki & Glenn (1961) found that although 6-MAP was twenty-five times more potent than progesterone in delaying parturition in the rabbit, it was without effect on uterine contractility *in vitro*.

The effectiveness of the intra-uterine administration route is further indicated by the myometrial response to intra-uterine oxytocin. It is not surprising that the intra-uterine dose required was at least 10 times the intravenous threshold dose, considering that rapid intravenous injection of a threshold dose may give rise to only one contraction, whereas intra-uterine injection, which results in slower absorption, produces an increased level of activity lasting 20 min or more. In contrast, mono-iodoacetate, which readily nullifies *in vitro* activity, was without effect even in high concentrations when injected into the uterine segment.

The results with progesterone, oxytocin and mono-iodoacetate illustrate

the importance of the integrity of the circulation, which may contain inactivating systems capable of neutralizing the mono-iodoacetate without interfering with the physiological actions of oxytocin.

The isotope determinations show that progesterone was, in fact, reaching the myometrium during the 30 min after its injection into the uterine segment, although the total found in the muscle layer of the segment amounted to only about 1% of the quantity injected. The concentration was, however, greater than in the endometrium in all three cases, which suggests that there may be an active absorption of progesterone from the uterine lumen rather than a passive diffusion.

In contrast to these findings, isotope determinations of the uptake of progesterone by the isolated uterine segment from the surrounding Krebs's solution (E. M. Glenn, personal communication) indicate that much greater concentrations are achieved *in vitro* than in the intact animal. However, if the bath fluid is blood plasma or if 4% albumen is first added to the Krebs's solution, the amount of progesterone absorbed by the segment is reduced to a fifteenth. Furthermore, under these circumstances the acute effect of progesterone-like compounds in reducing myometrial tension is no longer apparent. This would explain, therefore, why adequate concentrations of progesterone in the myometrium are not achieved in the living animal and why an acute depressant action cannot be demonstrated. The blood supply is apparently protecting the myometrium from high concentrations of progesterone, but exactly how this is achieved or what part this mechanism plays in the control of myometrial function by endogenous progesterone (Csapo, 1956; Schofield, 1961) is a matter for further investigation.

SUMMARY

1. The acute effect of progesterone on myometrial tension has been reinvestigated in the intact rabbit using an improved technique. Some related compounds have also been tested.

2. A depressant action could not be demonstrated in the intact rabbit when the compounds were given by intra-uterine injection.

3. The effectiveness of intra-uterine injection was indicated by the stimulant action of oxytocin injected in this way.

4. Experiments with progesterone 4-^{14}C isotope showed that progesterone was, in fact, reaching the myometrium of the intact uterus but in much lower concentrations than when isolated strips are suspended in Krebs's solution.

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REFERENCES

CSAPO, A. (1954). Dependence of isometric tension and isotonic shortening of uterine muscle on temperature and on strength of stimulation. *Amer. J. Physiol.* **177**, 348–354.

CSAPO, A. (1955). *Modern Trends in Obstetrics and Gynaecology.* 2nd series. London: Butterworth.

CSAPO, A. (1956). Progesterone 'block'. *Amer. J. Anat.* **98**, 273–292.

SCHOFIELD, B. M. (1955). The influence of the ovarian hormones on myometrial behaviour in the intact rabbit. *J. Physiol.* **129**, 289–304.

SCHOFIELD, B. M. (1957). The hormonal control of myometrial function during pregnancy. *J. Physiol.* **138**, 1–10.

SCHOFIELD, B. M. (1960). Hormonal control of pregnancy by the ovary and placenta in the rabbit. *J. Physiol.* **151**, 578–590.

SCHOFIELD, B. M. (1961). *Brook Lodge Symposium, Progesterone*, pp. 45–48. Kalamazoo, Michigan: Brook Lodge Press.

STUCKI, J. C. & GLENN, E. M. (1961). *Brook Lodge Symposium, Progesterone*, pp. 25–36. Kalamazoo, Michigan: Brook Lodge Press.

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HYPERGLYCAEMIA INDUCED BY DRUGS

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This paper concerns hyperglycaemia produced by drugs injected into the cannulated lateral cerebral ventricle, or intracisternally. Most experiments were performed on rabbits, but a few were done on cats. A difference between the two species was found.

In rabbits intraventricular injection of a small dose of adrenaline, barbitone, or leptazol resulted in an increase in blood glucose concentration. The hyperglycaemia produced by adrenaline could be accounted for by its absorption from the cerebrospinal fluid (c.s.f.) into the blood stream; rabbits are particularly sensitive to the metabolic effects of adrenaline (Cori, Cori & Buchwald, 1930). On the other hand, injection of barbitone or leptazol induced a hyperglycaemia of central origin mediated by the sympathetic nervous system.

In cats, which are relatively insensitive to the metabolic effect of adrenaline, the intraventricular injection of adrenaline in a dose larger than that effective in rabbits did not produce hyperglycaemia, but after its intracisternal injection there was a pronounced and sustained hyperglycaemia. This effect has been described previously by Leimdorfer, Arana & Hack (1947), who produced hyperglycaemia by injecting intracisternally 50 μg adrenaline/kg. The same amount of adrenaline injected into a joint or intraperitoneally did not raise the blood glucose concentration, so they concluded that the hyperglycaemia was of central origin. They also concluded that the adrenaline was not absorbed into the blood stream, since it could be detected in the cerebrospinal fluid 16 hr after an intracisternal injection of 0·5 mg.

METHODS

Experiments on rabbits

Belgian lop-eared rabbits of 2–3 kg body weight were used. A Collison cannula was implanted permanently into the left lateral cerebral ventricle according to the procedure of Feldberg & Sherwood (1954). The stem of the cannula was shorter than that used for cats;

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the length between the end of the threaded portion and the orifice in the polythene sleeve was 4·5–5·0 mm. The hole in the skull was made with an electrically operated dental drill, and not, as in cats, with a hand drill; this modification was essential because of the curvature of the calvarium at the point of insertion of the cannula.

The implantation was carried out aseptically under open ether anaesthesia and premedication with atropine (4 mg/kg subcutaneously) and pentobarbitone sodium (25 mg/kg intravenously). After exposure of the left parietal bone through a paramedian skin incision about 2 cm long, a hole was bored in the skull, with a 2·5 mm coarse burr, 7 mm lateral to the mid point of the sagittal suture: the hole was reamed vertically with a hand-operated twist drill (No. 30); the thread was cut and the cannula was then inserted. It did not matter if the cannula was not quite vertical, as long as its depth of insertion was correct. After completion of the cannulation the skin was sutured with silk. An interval of at least 1 week was allowed between cannulation and the experimental use of the rabbit.

In the experiments in which the splanchnic nerves were divided, an interval of at least 4 days elapsed between cannulation and splanchnicotomy, which was performed aseptically under the same anaesthetics as the ventribular cannulation. The splanchnic nerves were exposed by the abdominal route, and cut cephalic to the adrenal glands; care was taken to ensure that the small branches to the adrenals were included in the section. An interval of at least 6 days was allowed between splanchnicotomy and experimental use of the animal.

All the observations on the effects of drugs were made without anaesthesia. Food was withdrawn 24 hr before the start of each experiment. The rabbits were usually confined in boxes, but when leptazol was injected the rabbits were taken out of the boxes and placed on the floor. The drugs were always injected in a volume of 0·1 ml./kg body wt. In order to obtain blood samples for glucose determination, a 0·9 mm diameter × 0·5 mm bore transparent polyvinylchloride cannula was passed into the superior vena cava through a prick in the marginal vein of the ear, usually the right ear, and left *in situ* for the duration of each experiment.

Post-mortem examination. After the experiments had been performed, the position of the tip of the implanted cannula was determined. The animals were killed by intravenous injection of pentobarbitone sodium 60 mg/kg, and immediately afterwards a solution of bromophenol blue, 0·2 mg = 0·1 ml./kg, was injected through the ventricular cannula. After removal of the parietal and occipital bones, successful implantation was characterized by the absence of dye from the exposed cortical surface, and by its presence in the fourth ventricle; when coronal sections of the brain were made the dye was found in the left lateral and third ventricles, and in the aqueduct; the right lateral ventricle was not regularly stained. The results to be described were obtained in rabbits in which the implantation of the ventricular cannula had been successful, and in which there was no evidence of blockage of the flow of c.s.f. Signs of obstruction were the presence of an old blood clot in the anterior part of the cannulated ventricle and a dilated left lateral ventricle.

In the rabbits in which splanchnicotomy had been performed, the splanchnic nerves were dissected in the thorax, and traced caudally to the site of section, to establish that not only the main trunk, but also the small branches, had been severed.

Experiments on cats

In cats anaesthetized with pentobarbitone sodium (33 mg/kg), a Collison cannula was implanted aseptically into the left lateral cerebral ventricle, according to the method described by Feldberg & Sherwood (1954). The observations on the effect of intraventricular, intracisternal or intraperitoneal injections were made under pentobarbitone sodium anaesthesia, at least 1 week after the cannulation; the volumes injected were 0·1 ml./kg body weight. For the intracisternal injections the hair on the back of the neck was clipped. A needle was inserted, without incision of the skin, through the atlantooccipital membrane, and on the appearance of a drop of clear c.s.f. the syringe containing the drug was attached,

and the injection was made, carefully avoiding movement of the needle. To obtain blood for glucose determination a soft polyethylene cannula was passed into the inferior vena cava through the saphenous vein, and left in place throughout the experiment.

Blood glucose was determined by the specific glucose oxidase method (Huggett & Nixon, 1957) using the enzyme reaction mixture as supplied by Boehringer & Soehne, Mannheim; 2·85 % (v/v) perchloric acid was used as the protein precipitant, and the incubation was carried out at room temperature for 40 min (Jakobsen, 1960).

Substances used for injection

Adrenaline and L-*noradrenaline bitartrate.* The salt was dissolved in NaCl solution 0·9 g/100 ml., immediately before use. In order to obtain a solution containing 20 μg adrenaline base/0·1 ml., 3·65 mg. of the bitartrate was dissolved in 10 ml.

Barbitone sodium. At the higher concentrations used (2 and 1·15 g/100 ml.) this drug is soluble only in an alkaline medium. The 2 % solution was made by dissolving 100 mg barbitone sodium in 3 ml. distilled water, adding 0·4 ml. 0·3N-HCl, and then making the volume up to 5 ml. with distilled water. The 1·15 % solution was prepared by mixing 27·7 ml. 0·1N barbitone sodium with 23·3 ml. 0·1N-HCl. The pH of the solution, measured with a glass electrode, was 7·5–7·6 for the 2 % and 7·0–7·2 for the 1·15 %, at which the barbitone remained in solution at room temperature for at least 20 min.

Leptasol was prepared as a solution, 1 g/100 ml. 0·9 % saline.

RESULTS

Experiments on rabbits

Adrenaline

When injected into the cerebral ventricle, 20 μg adrenaline/kg was sufficient to increase the glucose concentration of the blood. The peak concentration was reached after 1 hr. A typical experiment is illustrated in Fig. 1*A*, and Table 1 shows the mean values, of 12 experiments, of the blood glucose concentration before and at various times after the injection. The mean value of 83·5 mg/100 ml. immediately before the injection, rose to a maximum of 134·5 mg/100 ml. after 1 hr and then fell to 114 mg/ml. during the following hour.

Since the adrenaline was injected as bitartrate, the solution had a pH of 3·5. However, the effect of the injection was not due to its acidity, because intraventricular injection of an equivalent amount of tartaric acid, 16·4 μg/kg made up in 0·9 % NaCl solution, did not affect the blood glucose concentration (see line 2 of Table 1).

The effect of the adrenaline injection could not be ascribed to a central action mediated by the sympathetic nervous system, since it persisted after bilateral section of the splanchnic nerves. This is shown in the experiment in Fig. 1*A*, and in Table 1, which gives the mean blood glucose concentrations of twelve rabbits, injected with 20 μg adrenaline/kg intraventricularly after section of the splanchnic nerves. The mean blood glucose values before as well as after the injection were somewhat lower than those of the twelve rabbits with intact splanchnic nerves, but the response to the injection was much the same in both series of experiments.

Evidence that the splanchnic nerves had been successfully divided and that no functional regeneration had occurred was obtained by determining the effect on blood glucose of an intraventricular injection of leptazol (see page 132).

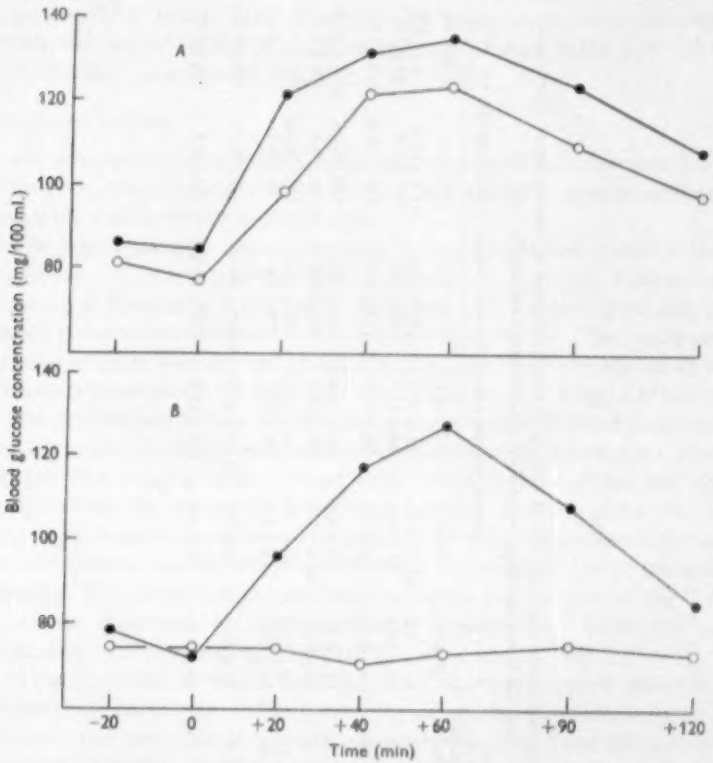

Fig. 1. Responses of blood glucose concentration in two unanaesthetized rabbits *A* to adrenaline, 20 μg/kg, and *B* to barbitone sodium, 2 mg/kg, each injected at zero time into the lateral cerebral ventricle. ●—● intact animal; ○—○ 6–8 days after bilateral splanchnicotomy.

The hyperglycaemia produced by the intraventricular injection of 20 μg adrenaline/kg could be accounted for by absorption of the adrenaline from the c.s.f. into the blood stream, since the same amount of adrenaline injected intraperitoneally caused a hyperglycaemia of about the same magnitude and time course as the intraventricular injection. This is shown in the fourth line of Table 1, which gives the mean values obtained from six experiments. These findings suggest that absorption of the adrenaline

TABLE 1. Blood glucose concentrations in unanaesthetized rabbits 20 min before and at various times after various injections into the lateral cerebral ventricle (I.VT.) or intraperitoneally (I.P.)

Drug given at zero time	No. of rabbits	Blood glucose concentrations (mean ± S.D.) mg/100 ml. at						
		−20 min	Zero	+20 min	+40 min	+60 min	+90 min	+120 min
Adrenaline, 20 μg/kg, I.VT.	12	85 ± 7·4	83·5 ± 6·1	111 ± 18·0	125 ± 15·4	134·5 ± 15·8	126 ± 16·4	114 ± 12·8
D-tartaric acid, 16·4 μg/kg, I.VT.	6	83 ± 10·3	84 ± 9·8	84 ± 11·1	83 ± 7·3	77·5 ± 6·2	84 ± 7·1	87·5 ± 7·2
Adrenaline, 20 μg/kg, I.VT. after bilateral splanchnicotomy	12	77·5 ± 9·5	74 ± 7·6	92 ± 21·0	112 ± 23·0	122 ± 13·6	118 ± 14·0	110 ± 11·9
Adrenaline, 20 μg/kg, I.P.	6	82 ± 6·2	81 ± 4·0	115 ± 10·3	128 ± 13·7	132 ± 25·8	122·5 ± 31·9	122 ± 35·4
L-noradrenaline, 20 μg/kg, I.VT.	6	82 ± 9·5	85·5 ± 7·5	93 ± 14·6	91 ± 9·5	89 ± 4·9	88 ± 4·2	93 ± 4·4
Barbitone sodium, 2 mg/kg, I.VT.	12	85 ± 15·7	81 ± 11·5	114 ± 16·5	122 ± 16·9	126 ± 15·6	106 ± 14·1	99 ± 14·5
Barbitone sodium, 2 mg/kg, I.VT. after bilateral splanchnicotomy	12	75 ± 6·3	74·5 ± 6·5	78 ± 8·8	79 ± 8·6	79 ± 8·6	78 ± 8·4	82 ± 9·1
Barbitone sodium, 1·15 mg/kg, I.VT.	6	81 ± 4·7	80 ± 5·4	111·5 ± 12·0	106·5 ± 12·9	99 ± 10·8	101 ± 9·2	104 ± 11·1
Sodium acetate, 0·1 M, pH 8·0, 0·1 ml./kg, I.VT.	6	89 ± 9·9	91 ± 7·8	115 ± 12·3	113 ± 12·3	104 ± 9·6	103 ± 7·2	105 ± 8·5

proceeds at about the same rate from the c.s.f. as from the peritoneal cavity.

Noradrenaline

This sympathomimetic amine, which is known to have a much weaker glycogenolytic action than adrenaline on systemic injection (Schümann, 1949), did not affect the blood glucose concentration when injected intraventricularly in a dose of 20 μg/kg.

Barbitone sodium

Intraventricular injection of 2 mg barbitone sodium/kg produced hyperglycaemia, vasodilatation of the external ear, sedation, ataxia, nystagmus, and with smaller doses hyperphagia.

The hyperglycaemia was comparable in magnitude and duration to that produced by an intraventricular injection of 20 μg adrenaline/kg. Figure 1*B* illustrates a typical experiment, and Table 1 gives the mean blood glucose concentrations of twelve experiments. The peak of the hyperglycaemia was reached about 1 hr after the injection, the mean blood glucose concentration having then risen from 81 to 126 mg/100 ml.

The mechanism of the barbitone hyperglycaemia differed from that of the adrenaline hyperglycaemia. The response was abolished after bilateral section of the splanchnic nerves, and was therefore of central origin, mediated by the sympathetic nervous system. Table 1 gives the mean blood glucose concentrations of twelve rabbits with both splanchnic nerves divided, before and after intraventricular injection of barbitone sodium 2 mg/kg. The difference in the effect of splanchnicotomy on the hyperglycaemia produced by intraventricular injection of barbitone or of adrenaline is illustrated by a comparison of *A* and *B*, Fig. 1.

In four rabbits, in which only the right splanchnic nerve was cut, the response to barbitone sodium 2 mg/kg injected intraventricularly was reduced: the mean blood glucose concentration rose from 75 mg/100 ml. before the injection to 98·5 mg/100 ml. 1 hr later.

The failure of intraventricularly injected barbitone to raise the blood glucose concentration after bilateral splanchnicotomy was not due to a post-operative deficit of liver glycogen demonstrable by failure to respond to adrenaline, since an intraperitoneal injection of 20 μg adrenaline/kg 2 hr after the barbitone produced a hyperglycaemic response. This control injection was given to six of the twelve rabbits referred to in Table 1, line 7, and in 30 min the mean blood glucose concentration rose from 80 to 123·5 mg/100 ml.

The injection of the barbitone sodium 2 mg/kg was in a 2% solution, which had to be alkaline to avoid precipitation: it had a pH of 7·5–7·6.

At this pH barbitone is a powerful buffer. These properties of the solution, however, did not fully account for the observed hyperglycaemia, as was shown by the following two observations:

(1) The intraventricular injection of a smaller dose of barbitone sodium (1·15 mg/kg), injected as a weaker solution (1·15 %), which was sufficiently stable at a pH of 7·0–7·2, also produced a hyperglycaemia, but the effect was less pronounced. As is shown in Table 1, in six experiments in which 1·15 mg/kg was injected intraventricularly the mean blood glucose concentration rose from 80 to 115·5 mg/100 ml. in 20 min, but there was no further rise such as occurred with the larger dose of barbitone.

(2) An intraventricular injection of 0·1 M sodium acetate solution, 0·1 ml/kg, made alkaline to pH 8·0 with NaOH, increased the blood glucose concentration, but the increase was less than after 2 mg barbitone/kg: it was comparable to that after 1·15 mg/kg (see Table 1). The injection of this alkaline solution also failed to produce nystagmus or sedation.

Vasodilatation of the ear occurred within 1 min of the injection of 2 mg/kg, and persisted for about 1 hr. With smaller doses (0·25–0·5 mg/kg) the onset of the vasodilatation occurred a little later, and its duration was about 30 min.

Sedation and ataxia, which preceded the onset of the nystagmus, became pronounced within 15–20 min and decreased gradually about 1 hr after the intraventricular injection of 2 mg barbitone sodium/kg. At the height of the effect, the animal made no movements as long as it was left undisturbed. When lying on its belly, the rabbit could be easily turned on its side, without resistance; it then made no effort to return to its former position. The rabbit could also be placed on its back, and if lightly supported retained this position, even on further handling. The hind legs could be passively extended and flexed without eliciting noticeable muscular resistance, but a prompt withdrawal reaction was observed on pinching the paws. Some rabbits did not respond to sudden noise, others lifted the head momentarily. When induced to move profound ataxia was revealed, and the rabbit fell on its side.

Nystagmus began within 5–8 min of the injection of 2 mg/kg and lasted for up to 20 min. The direction of the nystagmus depended on the position of the rabbit's head. When the head rested on its side, the nystagmus was vertical with respect to the rabbit's head; when the head was upright the nystagmus was horizontal, with slow movements usually to the right, that is, away from the cannulated side. The nystagmus sometimes began at a low frequency which increased during the following 10 min; the maximal frequency attained in different animals lay between 20 and 80/min. At the

onset the nystagmus was easily suppressed by gentle handling of the animal, but later it was persistent.

Provided that the tip of the cannula was in the lateral ventricle and that the passage from the lateral to the third ventricle was not obstructed, the intraventricular injection of barbitone sodium regularly produced nystagmus. Whenever nystagmus failed to occur, either the cannula had perforated the floor of the lateral ventricle and lay in the subarachnoid space below the occipital cortex, or the outflow from the lateral to the third ventricle was impeded by old blood clot. As a routine test of proper cannulation, the effect of an intraventricular injection of 2 mg/barbitone sodium/kg was therefore always examined; animals which failed to show nystagmus were rejected from all experiments. When 1·15 mg barbitone sodium/kg was injected intraventricularly, the nystagmus lasted for a few minutes only.

Hyperphagia was seen after intraventricular injection of 0·25–0·5 mg barbitone sodium/kg, if the rabbits had access to food. They regularly went to their food boxes 3–7 min after the injection, and ate voraciously without pause for 6–12 min. With these small doses of barbitone neither nystagmus nor ataxia nor signs of sedation were seen. Following an intraventricular injection of 1–2 mg barbitone sodium/kg, the rabbits made vigorous chewing movements for 15–60 sec, but made no attempt to go to the food box; they sat perfectly still, and a few minutes later their heads sank on the forepaws.

Leptazol

Leptazol is a central excitor of the sympathetic nervous system, and is known to cause hyperglycaemia in rabbits when injected subcutaneously in a dose of 40 mg/kg (Bömer, 1930); on intraventricular injection a much smaller dose was effective. The intraventricular injection of 1 mg leptazol/kg caused, within a few seconds, violent excitation, characterized by unrestrainable rapid running in small circles, usually towards the cannulated side. This effect lasted for 5–10 min; during this period there was often urination and defaecation. The injection also increased the blood glucose concentration. This was shown in eight rabbits, in which the injection was made 2 hr after the intraventricular injection of 2 mg barbitone/kg. At the time of the leptazol injection the mean blood glucose concentration was 94·5 mg/100 ml. Thirty minutes later it had risen to 119·5 mg/100 ml. In rabbits with their splanchnic nerves divided this increase did not occur, although the behavioural changes persisted.

These results show that the hyperglycaemia is brought about by a central action of leptazol and is mediated by the splanchnic nerves. Leptazol can therefore be used to test the success of splanchnicotomy and

the absence of functional regeneration. This test was applied to each of the twelve rabbits with divided splanchnic nerves, in which the intraventricular injection of 20 μg adrenaline/kg had produced a hyperglycaemia. In these rabbits an intraventricular injection of 1 mg leptazol/kg, given 2 hr after the adrenaline, when the mean blood glucose concentration had fallen to 110 mg/100 ml., did not increase the blood glucose concentration, which continued to fall to 102 mg/100 ml. during the following 30 min.

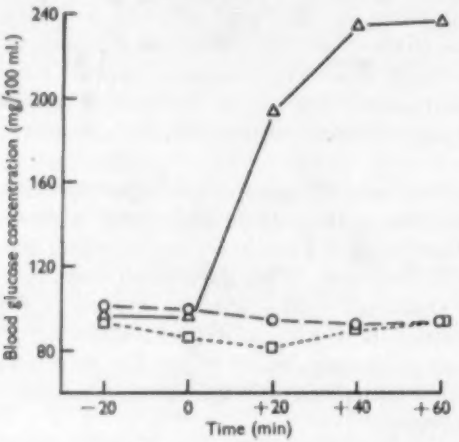

Fig. 2. Responses of blood glucose concentration in three cats, anaesthetized with pentobarbitone sodium, to adrenaline 62·5 μg/kg injected at zero time by different routes: ○—○ intraperitoneally; □—□ intraventricularly; △—△ intracisternally. Ordinate: time in min. Abscissa: blood glucose concentration, in mg/100 ml.

When only the right splanchnic nerve had been divided, leptazol still increased the blood glucose concentration, and the response was not much smaller than with both splanchnic nerves intact. In the four rabbits with the right splanchnic nerve divided, in which the mean blood glucose concentration had risen from 75 to 98·5 mg/100 ml. 1 hr after the intraventricular injection of 2 mg barbitone sodium/kg (see p. 129), the mean blood glucose concentration fell 1 hr later to 92 mg/100 ml. At this time 1 mg leptazol/kg was injected intraventricularly, and 30 min later the mean blood glucose concentration rose to 112·5 mg/100 ml.

Experiments on cats

The few experiments so far carried out on cats anaesthetized with pentobarbitone sodium have given constant results. Adrenaline 62·5 μg/kg, injected either intraventricularly or intraperitoneally, did not affect the

blood glucose concentration; but the same dose, injected intracisternally, induced a pronounced and sustained hyperglycaemia. These results are illustrated in Fig. 2. The response to the intracisternal injection of adrenaline was not brought about by injury of the medulla, comparable to the piqûre of Claude Bernard (1858), nor by the low pH of the injected solution, since the intracisternal injection of a more acid solution, tartaric acid 0·25 mg = 0·1 ml/kg, did not affect the blood glucose concentration.

DISCUSSION

The hyperglycaemia in unanaesthetized rabbits after the injection of a small dose of adrenaline into the cannulated lateral cerebral ventricle is considered not a central but a peripheral action of adrenaline, following its absorption into the blood stream. Bilateral splanchnicotomy does not abolish the hyperglycaemia after the intraventricular injection of adrenaline. The failure of noradrenaline, injected into the cerebral ventricle, to produce hyperglycaemia is also consistent with this conclusion, because systemically noradrenaline was only 1/20th of the hyperglycaemic potency of adrenaline in the rabbit (Schûmann, 1949). Since the magnitude and time course of the hyperglycaemia produced by the intraventricular injection of 20 μg adrenaline/kg resembles that observed after an intraperitoneal injection of the same dose, absorption, at least of small doses of adrenaline, from the c.s.f. is envisaged to occur at approximately the same rate as from the peritoneal cavity.

Unlike the hyperglycaemia produced by adrenaline in unanaesthetized rabbits, that following the intraventricular injection of a small dose of barbitone sodium or of leptazol is due to a central action of these drugs. Part of the effect of barbitone may be attributed to the alkalinity of the solution injected and its buffering. These centrally induced hyperglycaemias are mediated by the splanchnic nerves. They are not observed after bilateral splanchnicotomy, when intraperitoneal adrenaline still produced its hyperglycaemic response. Barbiturates are central depressants (Tatum, 1939), while leptazol is a central excitant and an antagonist of the barbiturates (Thorp, 1947). The fundamental question therefore arises, is the centrally induced barbitone hyperglycaemia a manifestation of an excitatory or of a depressant action? If the latter, this would suggest release of a sympathetic centre from an inhibitory influence.

The hyperphagia seen in rabbits after the intraventricular injection of small doses of barbitone recalls similar observations on cats given chloralose, chloral, urethane, $MgCl_2$, or pentobarbitone (Feldberg, 1958). These substances produced hyperphagia on intraventricular injection; with chloralose the effect was also seen after intravenous injection in subanaesthetic doses. The hyperphagia was associated with profound ataxia, whereas in our

experiments on rabbits hyperphagia occurred only with doses of barbitone too small to cause ataxia. With larger doses, which made the rabbits ataxic, the only remnant of a possible hyperphagia was the vigorous chewing movements. Feldberg (1959) attributed the hyperphagia in cats to a depressant action of the anaesthetic and hypnotic agents on the ventromedial nuclei of the hypothalamus. These nuclei are known to exert an inhibitory control over the more lateral areas of the hypothalamus, which are considered to be the actual 'feeding centres' (Anand & Brobeck, 1951). The same explanation would account for the hyperphagia observed in the rabbit, but it would appear that in this species, the ventromedial nuclei are more sensitive or more accessible to the intraventricularly injected barbitone than the neural structures concerned in the production of ataxia.

It is common knowledge that in rabbits an intravenous injection of pentobarbitone may cause a slight transient nystagmus. In contrast, the nystagmus after an intraventricular injection of 2 mg barbitone sodium/kg is severe and prolonged. Structures beneath the lining of the lateral ventricle can be excluded as the sole site of action of barbitone in producing this effect, since nystagmus does not occur if the passage from the lateral to the third ventricle is obstructed. The barbitone probably acts on structures beneath the lining of the third ventricle and the aqueduct; penetration to some depth would account for the 5–8 min latency between the injection and the onset of nystagmus.

The alteration of the direction of the nystagmus on rotation of the rabbit's head is consistent with the conclusion of Lorente de Nó (1933) that nystagmus is a rhythmic reflex conditioned by postural labyrinthine stimuli. Szentagothai (1950) has emphasized that many of the vestibulo-ocular reflex arcs pass through the reticular formation, which plays an essential role in inhibitory responses. The nystagmus following intraventricular injection of barbitone may therefore be a release of the rhythmic reflex from the inhibitory influences of its reticular connexions, by depression of structures in the diencephalic and mesencephalic reticular formation. Depression of structures in these regions may also be the cause of the ataxia and sedation seen after the intraventricular injections of barbitone.

From their experiments on cats Leimdorfer *et al.* (1947) concluded that adrenaline was not absorbed from the c.s.f. because it could be detected in this fluid several hours after intracisternal injection; furthermore, they did not observe a rise in blood pressure after the injection. However, an increase in blood pressure would not be expected if the rate of absorption were slow. In cats no quantitative data are available for the absorption of adrenaline from the c.s.f., and without such data it is not possible to state whether the conditions of absorption differ from those in the rabbit. This

seems unlikely, particularly since histamine is absorbed from the c.s.f. of the cat at a rate about half that from subcutaneous tissue (Bhawe, 1958).

To exert a glycogenolytic action in the cat, much larger amounts of adrenaline would require to pass from the c.s.f. into the blood stream than in the rabbit. In fact, hyperglycaemia was not obtained in the cat after the intraventricular injection of three times the dose given to the rabbits. However, when the adrenaline was injected intracisternally it did produce hyperglycaemia, which confirms the observation of Leimdorfer *et al.* (1947). As already inferred by these authors, this hyperglycaemia is not due to a peripheral action of adrenaline. The adrenaline must act on some structures in the brain which are reached in sufficient concentration by injection through the atlanto-occipital membrane, but not by injection through the cannulated lateral cerebral ventricle. The location of these structures is not known. One possibility is the area postrema, which is known to contain large amounts of noradrenaline (Vogt, 1954) and to be sensitive to the central emetic action of adrenaline (Borison, 1959). Another possibility is that adrenaline injected intracisternally reaches the floor of the fourth ventricle in much higher concentrations than when injected intraventricularly, and acts on structures in this region, perhaps those involved in the piqûre diabetes of Claude Bernard (1858). Yet another possibility is that the site of action of the adrenaline is outside the fourth ventricle, in some superficial area of the medulla oblongata or of the upper cervical cord. This problem requires further investigation.

SUMMARY

1. In unanaesthetized rabbits adrenaline 20 μg/kg injected into the lateral cerebral ventricle produced hyperglycaemia, which was not abolished by bilateral splanchnicotomy, and which was attributed to absorption of the adrenaline from the c.s.f. into the blood stream. The rate of absorption was comparable to that from the peritoneal cavity, since a similar hyperglycaemia was obtained after intraperitoneal injection of the same dose of adrenaline.

2. In unanaesthetized rabbits the intraventricular injection of L-noradrenaline 20 μg/kg had no effect on blood glucose.

3. In unanaesthetized rabbits intraventricular injection of barbitone sodium 0·25–0·5 mg/kg caused vasodilatation of the external ear and hyperphagia; the intraventricular injection of 2 mg/kg produced flushing of the ears, sedation, ataxia, nystagmus, and hyperglycaemia. The hyperglycaemia was due to a central action; it did not occur after bilateral splanchnicotomy.

4. In unanaesthetized rabbits the intraventricular injection of leptazol

1 mg/kg produced a hyperglycaemia of central origin which was abolished by bilateral splanchnicotomy.

5. In cats, anaesthetized with pentobarbitone sodium, a dose of adrenaline 62·5 μg/kg did not produce hyperglycaemia when injected either into the cannulated lateral cerebral ventricle or intraperitoneally, but induced hyperglycaemia when given intracisternally, suggesting an action on some structures which are reached in sufficient concentration by this route but not by intraventricular injection.

REFERENCES

ANAND, B. K. & BROBECK, J. R. (1951). Hypothalamic control of food intake in rats and cats. *Yale J. Biol. Med.* **24**, 122–140.

BERNARD, C. (1858). *Leçons sur la Physiologie et la Pathologie du Système Nerveux.* Vol. 1, pp. 397–447. Paris: Baillière.

BHAWE, W. B. (1958). Experiments on the fate of histamine and acetylcholine after their injection into the cerebral ventricles. *J. Physiol.* **149**, 169–189.

BÖMER, M. (1930). Über die Wirkung einiger Krampfgifte auf Blutzucker und Blutmilchsäure. *Arch. exp. Path. Pharmak.* **149**, 247–256.

BORISON, H. L. (1959). Effect of ablation of medullary emetic chemoreceptor trigger zone on vomiting responses to cerebral intraventricular injection of adrenaline, apomorphine and pilocarpine in the cat. *J. Physiol.* **147**, 172–177.

CORI, C. F., CORI, G. T. & BUCHWALD, K. W. (1930). The mechanism of epinephrine action. VI. Changes in blood sugar, lactic acid, and blood pressure during continuous intravenous injection of epinephrine. *Amer. J. Physiol.* **93**, 273–283.

FELDBERG, W. (1958). Anaesthesia and sleep-like conditions produced by injections into the cerebral ventricles of the cat. *J. Physiol.* **140**, 20–21*P*.

FELDBERG, W. (1959). A physiological approach to the problem of general anaesthesia and of loss of consciousness. *Brit. med. J.* ii, 771–782.

FELDBERG, W. & SHERWOOD, S. L. (1954). Injections of drugs into the lateral ventricle of the cat. *J. Physiol.* **123**, 148–167.

HUGGETT, A. ST G. & NIXON, D. A. (1957). Enzymic determination of blood glucose. *Biochem. J.* **66**, 12*P*.

JAKOBSEN, L. K. (1960). Quantitative determination of blood glucose using glucose oxidase and peroxidase. *Scand. J. clin. Lab. Invest.* **12**, 76.

LEIMDORFER, A., ARANA, R. & HACK, M. H. (1947). Hyperglycaemia induced by the action of adrenaline on the central nervous system. *Amer. J. Physiol.* **150**, 588–595.

LORENTE DE NÓ, R. (1933). Vestibulo-ocular reflex arc. *Arch. Neurol. Psychiat.* **30**, 245–291.

SCHÜMANN, H. J. (1949). Vergleichende Untersuchungen über die Wirkung von Adrenalin, Arterenol und Epinin auf Blutdruck, Milzvolumen, Darm und Blutzucker. *Arch. exp. Path. Pharmak.* **206**, 164.

SZENTAGOTHAI, J. (1950). The elementary vestibulo-ocular reflex arc. *J. Neurophysiol.* **13**, 395–407.

TATUM, A. L. (1939). The present status of the barbiturate problem. *Physiol. Rev.* **19**, 472–502.

THORP, R. H. (1947). The comparison of respiratory stimulant drugs. *Brit. J. Pharmacol.* **2**, 98–99.

VOGT, M. (1954). The concentrations of sympathin in different parts of the central nervous system under normal conditions after the administration of drugs. *J. Physiol.* **123**, 451–481.

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CORTICOSTEROIDS IN PLASMA AND CELLS OF ADRENAL VENOUS BLOOD

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Investigations by several authors appeared to show that no appreciable amounts of corticosteroids are present in the cell fraction of adrenal venous blood (Bibile, 1953) or in peripheral human blood (Eik-Nes, Nelson & Samuels, 1953; Migeon, Sandberg, Decker, Smith, Paul & Samuels, 1956; Bush, 1957). This view has been generally accepted and as a result most of the estimates of the secretory capacity of the adrenal cortex have been carried out by studying the steroid content of the plasma alone. There are, however, hints in the literature that corticosteroids might become associated with the blood cells (Peterson, Wyngaarden, Guerra, Brodie & Bunim, 1955; Bush, 1957).

Studies on the secretion rate of aldosterone are made difficult by the very low concentrations of this steroid in adrenal venous plasma. It seemed, therefore, desirable to examine the possibility that some aldosterone was associated with the cells. A sample of adrenal venous blood was divided into two equal portions. One of these was centrifuged and the plasma and cells were extracted separately. The other portion was extracted as whole blood. The experiment showed that some aldosterone was, in fact, extracted from the cells, so that by using plasma alone a valuable source of aldosterone was wasted. It also showed that the estimate of aldosterone in the whole-blood sample was approximately three times as large as the sum of the estimates of aldosterone in the separated plasma and cell fractions of the same blood. This suggested that the method of extraction used broke down when applied to the closely packed cell fraction.

These observations have been followed up and the experiments have been extended to include estimations of corticosterone and cortisol.

METHODS

Operative procedures

Mongrel dogs were anaesthetized with ether followed by chloralose (70 mg/kg I.V.). The left adrenolumbar vein was exposed by an abdominal mid-line incision and cannulated

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lateral to the adrenal gland, after tributaries other than those coming from the adrenal gland had been tied. Heparin (500 u./kg) was injected intravenously. The adrenal vein was ligated at its point of entry into the vena cava. Adrenal venous blood was collected over periods of 20–150 min in polythene centrifuge tubes or glass cylinders immersed in ice-water.

When homologous blood or dextran was infused in the course of the experiment the dog received a preliminary intravenous injection of mepyramine maleate B.P. 5 mg/kg. This was done in order to try to prevent sudden falls in blood pressure and loss of plasma or dextran into the tissues. Bliss, Johns & Burgen (1959) and Remington & Baker (1959) have reported that dogs are often intolerant of homologous blood; oedema, loss of plasma from the circulation and prolongation of bleeding and clotting times may result from such infusions. Some of these effects are antagonized by mepyramine, but the improvement achieved is very variable.

Rat blood was collected under pentobarbitone anaesthesia (1% solution, 45 mg/kg, I.P.), guinea-pig blood under sodium barbitone (15% solution, 600 mg/kg, I.P.), rabbit blood under urethane (25% solution in 0·9% NaCl, 7 ml./kg, I.V.).

Rat and guinea-pig adrenal venous blood was collected from the left renal vein after tying off the renal pedicle and all veins not coming from the adrenal. The guinea-pig was previously eviscerated. The rabbit was eviscerated and blood was collected from the inferior vena cava caudal to the entry of the right and left renal veins. The renal veins and arteries and the abdominal aorta were tied and the vena cava was occluded at the level of the diaphragm (Vogt, 1955).

Chemical procedures

Solvent purification. Ethyl acetate was shaken once with a 1% solution of sodium carbonate and twice with water, dried over $CaCl_2$ and distilled twice. Methanol was distilled once from solid NaOH and redistilled. Ether was redistilled immediately before use.

Extraction. One volume of blood or plasma was diluted with one volume of water and extracted three times by shaking vigorously with two volumes of a mixture of ethyl acetate-ether 2:1 (v/v). Blood cells were first extracted in the same way. When this proved unsatisfactory they were diluted with three volumes of water instead of one. The amount of ethyl acetate-ether mixture used for each extraction was then four times the volume of the packed cells.

Purification of extracts. The purification procedure was similar to that described by Bush (1952) and by Holzbauer (1957). The combined extracts were washed consecutively with 1/8 of their calculated volume of a 0·2 N solution of Na_2CO_3, water and acidified water (10 drops of glacial acetic acid/100 ml.). The combined washings were twice re-extracted with 1/4 of their volume of the ethyl acetate-ether mixture. The washed extracts were dried with Na_2SO_4, decanted off and evaporated to dryness under reduced pressure at 50° C. For this purpose the flask was clamped into a device which was kept in slow rotating motion by a motor. The ethyl acetate-ether used to re-extract the washing solutions was poured over the Na_2SO_4 deposit, decanted and added to the main extract for evaporation. The dry residue of the combined extracts was then freed from fats by distribution between petroleum ether (b.p. 40–60° C) and ethanol. The volume of petroleum ether was 60 ml.; it was extracted once with 6 ml. and three times with 3 ml. of 80% ethanol. The ethanol was evaporated *in vacuo* in two portions from a 150 ml. round-bottom flask, with fresh petroleum ether added to reduce the often very troublesome frothing. The phospholipids were precipitated from acetone with $MgCl_2$, precisely as described before (Holzbauer, 1957).

Chromatography

All chromatograms were run at 27° C on Whatman No. 2 paper. The steroid spots were located on the developed papers by scanning in front of an U.V. lamp emitting at 240 mμ. The papers carried markers of pure steroids on each side.

Aldosterone, first chromatogram. After evaporation of the acetone the extract was dissolved

in 2 ml. ethanol and 1/20 of this solution removed for the estimation of cortisol and corticosterone. The remainder was evaporated to dryness, dissolved in 0·4 ml. dichloromethane and applied along a 5 cm horizontal line to a paper sheet, 22·5 cm wide. Two washings of 0·2 ml. CH_2Cl_2 were applied to the same region. A descending chromatogram was then run in the E_2B system of Eberlein & Bongiovanni (1955) which consists of petroleum ether (b.p. 80–100° C): tertiary butanol: water, 417:208:375 (v/v). In this system corticosterone has the highest R_F value, followed by cortisone and cortisol. The R_F value of aldosterone is somewhat smaller still than that of cortisol. Adrenal vein blood, however, contains so much cortisol that it invades the aldosterone region. (If 400 μg cortisol and 10 μg aldosterone were applied in this way, making sure that the area in which the two steroids were applied did not exceed 1 cm in the direction of the flow of the solvents, the small aldosterone spot could be clearly distinguished from, though it was touching, the large spot of cortisol.) For elution, rectangles of about 9 × 12 cm containing the aldosterone, contaminated by cortisol, were cut from the paper.

Aldosterone, acetylation. In order to separate aldosterone from cortisol the eluate was acetylated. The dried residue of the eluate was dissolved in 0·04 ml. of pyridine (purified by refluxing over BaO and distilling twice), 0·16 ml. of acetic anhydride was added and the samples were incubated for 2 hr in a water-bath at 60° C. Under these conditions aldosterone is transformed into its diacetate, whereas cortisol forms only a monoacetate, which is much more polar than the aldosterone diacetate. After acetylation the reagents were driven off *in vacuo*, care being taken that no traces were left behind.

Aldosterone, second chromatogram. The residues of the acetylation were dissolved in 0·3 ml. followed twice by 0·15 ml. of dichloromethane, and applied to paper. On each sheet there were two 8 cm lanes for the samples, and on each side a lane of 2·5 cm for the markers. The lanes were separated by 0·5 cm cut out strips. The samples were applied to the second 2 cm of the 8 cm starting line and run in a modified Bush B_2 system. The original B_2 system separates aldosterone diacetate and cortisol monoacetate well, but was sometimes found too fast for an over-night run. The speed of travel of the aldosterone diacetate was therefore decreased by changing the ratio of the solvents slightly in favour of the petroleum ether. The mixture finally used consisted of petroleum ether: benzene: methanol: water, 375:125:400:100 (v/v). In this system the spots of aldosterone diacetate are usually wide and large areas of the paper (8 × 9 cm) have to be eluted for quantitative recovery. This would cause high paper blanks if the final colorimetry were done on these eluates. Furthermore, small amounts of substances reacting with 'blue tetrazolium' were sometimes present close to the aldosterone diacetate region. Therefore the eluate of the aldosterone diacetate region was subjected to a third chromatographic procedure.

Aldosterone, third chromatogram. The eluates of the B_2 chromatogram were evaporated and the residues dissolved in 0·3 ml., followed twice by 0·15 ml. of dichloromethane and applied to washed papers (see below) as 1 cm^2 spots on 3 cm wide lanes which were separated by 0·5 cm cut out strips. Each sheet of paper consisted of seven such lanes; one at each end was used for the markers and two or three lanes served as paper blanks. Care was taken that the extracts did not reach the edges of the lanes. In order to reduce the blanks, papers used for this chromatogram were washed for 72 hr at room temperature. They were set up in tanks as for a descending chromatogram. For 24 hr the mobile phase of the modified A system (see below) was allowed to run over them. They were then transferred to a second tank where they were washed with a mixture of ethyl acetate-methanol 2:1 (v/v) for 48 hr. This is the mixture used for all elutions.

Bush's system A was used for the third chromatogram, but a little benzene was added in order to speed it up. The mixture thus consisted of petroleum ether (b.p. 80–100° C): benzene: methanol: water, 475:25:400:100 (v/v). During a 40 hr run in this system the aldosterone diacetate usually travelled 8–10 cm from the origin. The spots were discrete and not more than 20 cm^2 of paper had to be eluted. An additional advantage of running a third chromatogram is that the order in which the acetylated steroids run is not the same as in the

preceding system. In the B_2 system aldosterone diacetate runs a little faster than corticosterone monoacetate, whereas in the A system it runs more slowly. Though corticosterone monoacetate is not present in the extracts (corticosterone having been wholly removed in the first chromatogram), other impurities may behave in a similar way. We were, in fact, able to find occasionally an U.V.-absorbing substance contaminating the aldosterone region in the second chromatogram, but well separated from the aldosterone in the third. This substance did not react with blue tetrazolium.

Corticosterone and cortisol. Samples of up to 10 ml. of blood, plasma or cells of all species were extracted as described earlier for aldosterone. The residues of the final acetone extracts were applied to paper dissolved in 0·4 ml. dichloromethane. Whenever large samples of adrenal vein blood of the dog were worked up for aldosterone, one twentieth of the last ethanolic extract (see p. 138) was applied to paper for the estimation of cortisol and corticosterone. Each solution was applied in a 1 cm^2 area to 2 cm lanes, separated by $\frac{1}{2}$ cm cut out strips. Every sheet of paper had one or two blank lanes. On the two outermost lanes 10 μg control spots of cortisol and corticosterone were applied. A descending chromatogram was run in the solvent system, methanol:water:benzene, 275:225:500 (v/v) (B_2a; Bush & Sandberg, 1953).

Elution

A solvent mixture which is used to elute a substance from paper will be most efficient if the substance to be eluted travels with the front of the solvent mixture. Corticosteroids do this in a mixture of ethyl acetate-methanol 2:1 (v/v). This mixture was used for all elutions.

Aldosterone. The paper strips to be eluted from the aldosterone chromatograms were too large for the tubular eluters (see below). Therefore one edge of each paper strip was cut to a point. The other edge was inserted to a depth of 0·5–1 cm between two glass slides held together by a cotton or nylon thread. Rubber bands have to be avoided. The protruding portion of the paper strip was bent sharply over the edge of the slides, so that it hung vertically when the glass slides were stood in small glass troughs containing the eluting solvents. The eluate was collected in 10 ml. test tubes with ground-glass necks. The elution troughs and collecting tubes were fixed to stands which were placed in glass tanks. The walls of these tanks were lined with thick blotting paper which was soaked in the eluting mixture. The lid of the tank was sealed on with glycerine–starch paste. In order to achieve speedy and complete elution the tanks have to be saturated with the solvent. As the steroids travel with the front of the elution mixture, more than 80% are contained in the first drop of eluate. The elutions were usually allowed to run for 2 hr, when about 0·5 ml. of solvent had collected. Before putting the stopper on the elution tubes the necks had to be washed down carefully.

For recovery experiments quantities between 0·2 and 10 μg of different steroids were applied on 9×5 cm^2 paper strips and eluted. In thirty observations a mean recovery of 92% was obtained.

Corticosterone and cortisol. 6–7 cm long strips were cut out and eluted in small tubular glass eluters devised by Saffran & Sharman (1960).

Colorimetry

All steroids were estimated quantitatively by their colour reaction with blue tetrazolium in alkaline medium. The details for the determination of corticosterone and cortisol have been described previously (Vogt, 1955; Holzbauer & Vogt, 1957).

For the estimation of small amounts of aldosterone present in adrenal venous blood of the dog this method had to be modified by reducing the amount of some of the reagents. The dry residues of the final eluates were dissolved in 0·45 ml. of 95% ethanol (prepared to be free from aldehydes) and 0·04 ml. of a freshly made 1·25% solution of tetramethyl- or tetraethyl-ammonium hydroxide in 95% ethanol was added, followed by 0·05 ml. of a

freshly prepared 0·05% solution of blue tetrazolium (Gurr) in 95% ethanol. The solutions were well mixed, the tubes stoppered, and incubated in the dark for 1 hr at 23·5° C in a water-bath. The reaction was interrupted by adding 0·04 ml. of a 10% solution of acetic acid in 50% ethanol. The intensity of the developed colour was read against ethanol in the microcells of a Unicam spectrophotometer at a wave-length of 520 mμ. A standard solution was prepared from DL-aldosterone monoacetate (Ciba, ampoules of 1 mg/ml. 95% ethanol) by further acetylation to the diacetate. The contents of one ampoule were evaporated to dryness, taken up in 0·1 ml. of pyridine followed by 0·4 ml. of acetic anhydride and incubated at 60° C for 2 hr; the reagents were then driven off *in vacuo*. The residue was dissolved in 1 ml. absolute ethanol and stored at −16° C. The aldosterone diacetate in this solution ran as one spot in two different chromatographic systems.

A standard curve was calculated from thirty observations obtained on different days with amounts of aldosterone ranging from 0·5 to 8 μg. A straight line was obtained which ran through the origin and gave the conversion factor y/x of 0·073. The standard deviation from this calculated line is ±0·018, which corresponds to ±0·25 μg aldosterone monoacetate. Within one set of observations on any one day, however, amounts differing by 0·25 μg can be distinguished with confidence, as it is the whole set of estimations which may shift somewhat from day to day. For the special purpose of comparing different samples from one animal, the error is smallest if all estimations are done on the same day. The reagent blanks were equivalent to about 0·23 μg.

The samples which were eluted from the third chromatogram were read against paper blanks taken from other lanes of the same sheet of paper. Samples which had to be compared with each other were preferably run on the same sheet. The mean intensity of the colour reaction given by 51 paper blanks corresponded to 1·1 μg of aldosterone monoacetate with a standard deviation of ±0·36 μg. This included the reagent blank. The blanks have recently been reduced to 0·8 μg by the use of glass-distilled water for the solvent mixtures. The mean difference between two blanks from two separate lanes of the same paper corresponded to 0·17 ± 0·17 μg of aldosterone monoacetate.

Identification

The identification of the aldosterone was limited to the R_F values in three different chromatographic systems and its sulphuric acid chromogen.

Recovery of steroids added to arterial blood

Aldosterone. For recovery experiments a sample of crystalline DL-aldosterone was dissolved in absolute ethanol and amounts containing 5 μg pipetted off into ampoules. These were freeze-dried, sealed and stored at −15° C. The following recovery experiments were carried out.

(1) Two samples of 5 μg aldosterone were acetylated and run in the modified B_2 and the modified A system. From both samples 4·5 μg were recovered.

(2) Two samples of 5 μg aldosterone were applied to paper, run in the E_2B system, acetylated and then run in the modified B_2 and in the modified A system. 4·5 and 4·1 μg were recovered.

(3) Blood was collected from the carotid artery of a dog under ether anaesthesia and kept in the refrigerator during the night. On the following morning it was well mixed and divided into six 100 ml. portions. Aldosterone 5 μg was added to two blood samples which were then extracted. Another two samples were first extracted and the aldosterone added to the unwashed ethylacetate extracts. The remaining two samples were used as blanks. No aldosterone was detected in these two samples. From the 5 μg aldosterone added to each of the other four samples 3 μg were recovered in each case. A similar experiment was carried out using three 100 ml. arterial blood samples of another dog. 5 μg aldosterone was added to two samples. The third was used as a control and no aldosterone was detected in it. Extrac-

tion, purification and chromatography of one sample were completed in 5 days. The sample was on paper for approximately 75 hr. 2·9 μg aldosterone was recovered. The final estimations for the other sample were carried out after 9 days, the extracts having been on paper for approximately 130 hr. Only 2·1 μg was recovered. Thus, losses of added aldosterone in the course of the chromatographic procedures are seen to be of the order of 15%, provided delays are avoided. 25% is lost in the preceding purification procedure. Evidence for losses of added aldosterone during the extraction was not obtained.

Cortisol and corticosterone. Recovery experiments for cortisol and corticosterone added to small amounts of plasma and extracts of adrenal tissue have been described previously (Vogt, 1955; Holzbauer, 1957). Two experiments were carried out in which 200 μg corticosterone and 200 μg cortisol were added to 200 ml. of dog's arterial blood. The samples were extracted and purified as described above and from each sample an aliquot of 1/20 was subjected to chromatography in Bush's system B_3a. 7·8 and 8·2 μg of cortisol and 8·2 and 10·3 μg of corticosterone were found; this is a mean recovery of 86%. The amounts of cortisol and corticosterone which can be expected in 1/20 of 200 ml. dog's arterial blood are below the threshold of the colorimetric method (< 1 μg).

Efficacy of the extraction as applied to adrenal venous blood

An experiment was carried out in which three different methods for the extraction of cortisol and corticosterone from a sample of dog's adrenal venous blood were compared. A 5 ml. portion was diluted with an equal volume of water and extracted three times with two volumes of the ethyl acetate-ether mixture. A second sample was diluted with three volumes of water and extracted three times with four volumes of the ethyl acetate-ether mixture. A third sample was diluted with one volume of water and extracted three times with two volumes of chloroform, shaking 100 times by hand as with ethyl acetate. The following estimates for cortisol (F) and corticosterone (B) were obtained:

Sample 1; 17·3 μg F and 9·2 μg B.
Sample 2; 18·3 μg F and 9·3 μg B.
Sample 3; 16·5 μg F and 8·5 μg B.

The chloroform extract was more difficult to handle because of the formation of emulsions. The results did not suggest that any of the modifications of the usual extraction procedure constituted an improvement.

Before analysis, the samples used for the experiment had been kept for 9 days at +4° C. Another sample of the same blood had been analysed (by the first method) on the day of collection. The amount of steroids found was 18·4 μg F and 9·3 μg B. Thus standing for 9 days at +4° C did not cause any appreciable loss of steroids.

In another experiment the possibility was tested that there was hydrogen bonding between steroids and blood proteins, which might prevent their extraction with organic solvents. To 87 ml. of adrenal venous blood of a dog 87 ml. of water was added, extraction was carried out as usual with three portions of ethyl acetate-ether, and aldosterone, cortisol and corticosterone were estimated in the extract. To the remaining blood-water mixture another 87 ml. of water was added, and the mixture acidified to pH 2 by the drop-wise addition of concentrated HCl with constant stirring. This mixture was extracted three times with 260 ml. ethyl acetate-ether and the combined extracts were prepared as usual for chromatography. The haemin formed by the addition of HCl was extracted with the organic solvents, but most of it was removed by washing the extracts twice instead of once with 1% Na_2CO_3. The bulk of the extract was used for the determination of aldosterone, and 1/20 for the estimation of cortisol and corticosterone. In the neutral extract 3·45 μg aldosterone were found, and in the acid extract an additional 0·4 μg aldosterone. This figure lies at the threshold of the method and is not very accurate. In 1/20 of the neutral extract 11·5 μg cortisol and 5·9 μg corticosterone were found. None (< 1 μg steroid) could be detected in 1/20 of the acid extract. However, in the E_2B chromatogram of the larger por-

tion (19/20) of the acid extract which was used for the estimation of aldosterone, there were U.V.-absorbing spots which had the R_F values of cortisol, cortisone and corticosterone. Their intensity corresponded to approximately 10 μg steroid or 5–10 % of the content of the neutral extract. From these results it was concluded that acidifying the blood-water mixture before extraction with organic solvents would, at best, increase the steroid yield by 5–10%. As it was more difficult to purify such extracts it was decided not to acidify and to accept the small loss.

RESULTS

Corticosteroids in the cell fraction of adrenal venous blood of the dog

Adrenal venous blood was collected from four dogs (50, 51, 54 and 57, Table 1) over different periods of time into tubes kept in ice-water. At the end of the collection the blood of each dog was divided into two portions.

TABLE 1. Extraction of steroids from fractions of adrenal venous blood of the dog. One volume of water only was added to each fraction

Dog no.	Collection time (min)	Total blood collected (ml.)	Volume of fractions extracted individually (ml.)		Steroid found (μg)			% of calculated* amount extracted from cells		
					Aldosterone	Cortisol	Corticosterone	Aldosterone	Cortisol	Corticosterone
50	70	560	Whole blood	280	6·8	304	224	—	—	—
			Plasma	132	1·2	194	124	—	—	—
			Cells	148	1·0	126	90	18	114	90
51	70	312	Whole blood	156	5·0	284	236	—	—	—
			Plasma	70	0·7	106	116	—	—	—
			Cells	86	1·2	98	84	28	55	70
54	90	202	Whole blood	101	3·5	382	244	—	—	—
			Plasma	48	0·9	184	138	—	—	—
			Cells	53	0·2	168	122	8	85	115
57	75	363	Whole blood	121	3·2	354	184	—	—	—
			Plasma	113	2·9	364	208	—	—	—
			Cells	129	2·3	364	104	66	106	65

The volumes extracted as whole blood are one half (dogs 50, 51 and 54) or one third (dog 57) of the total.

* The calculated amount is the difference between the quantity of a steroid found in whole blood and that found in plasma.

One portion (one half of the total in dogs 50, 51 and 54, one third in dog 57) was extracted as whole blood. The other portion was centrifuged for 15 min ($g = 2200$) and plasma and cells were extracted separately. To each sample an equal volume of water was added and estimation of aldosterone, corticosterone and cortisol carried out as described under Methods. The results (Table 1) show that in all four dogs the three steroids were extracted not only from the plasma but also from the cells. The amounts of aldosterone extracted from the samples of whole blood were up to three times larger than the amounts extracted from the same volume of blood after its separation into plasma and cells. In the estimates of cortisol and corticosterone this discrepancy was smaller or absent.

The next step was to try and determine the reason why, as just shown, aldosterone seemed to be more easily extracted from cells diluted with plasma than from packed cells. In the first experiment of Table 2 (dog 53), packed cells from adrenal blood were diluted with arterial plasma. Of the 370 ml. adrenal venous blood collected, one third was extracted as whole blood; two thirds were centrifuged and 106 ml. plasma and 140 ml. cells obtained. Each of these fractions was divided into two equal portions.

TABLE 2. Extraction of steroids from fractions of adrenal venous blood of the dog. Comparison of yield from packed cells lysed with one or with several volumes of water

Dog no.	Collection time (min)	Total blood collected (ml.)	Volume of fractions extracted individually (ml.)		Volume of water (or diluent) added	Steroid found (μg)			% of calculated* amount extracted from cells		
						Aldosterone	Cortisol	Corticosterone	Aldosterone	Cortisol	Corticosterone
53	135	369	Whole blood	123	1	5·5	148	88	—	—	—
			Plasma	53	1	3·0	68	64	—	—	—
			Plasma	53	1	2·9	70	56	—	—	—
			Cells	70	1	1·0	42	36†	39	53	128†
			Cells	70	2·5‡	2·4	—	—	94	—	—
56	180	476	Whole blood	238	1	13·0	932	578	—	—	—
			Plasma	118	1	1·4	426	328	—	—	—
			Cells	60	1	3·4	118	100	59	47	80
			Cells	60	3	4·5	294	160	78	116	128
66	120	228	Whole blood	76	1	1·2	194	68	—	—	—
			Plasma	76	1	0·6	206	72	—	—	—
			Cells	76	3	1·8	214	72	100	117	112

The volumes extracted as whole blood are one half (dog 56) or one third (dogs 53 and 66) of the total.

* The calculated amount is the difference between the quantity of a steroid found in whole blood and that found in plasma. † values too near the threshold of the method to be accurate (glucocorticoids estimated in 1/20 of the whole extract). ‡ 53 ml. arterial plasma and 123 ml. water.

The whole blood sample (123 ml.), the two plasma samples (53 ml. each) and one cell sample (70 ml.) were extracted after the addition of equal volumes of water. To the second cell sample (70 ml.) 53 ml. of peripheral plasma of the same dog was added followed by 123 ml. of water. According to Table 2, $5{\cdot}5 - 3{\cdot}0\ \mu g = 2{\cdot}5\ \mu g$ aldosterone should have been found in the cell fraction, but only $1\ \mu g$ or 40% of the calculated quantity was extracted from the packed cells. When, however, the cells were diluted with plasma and a larger volume of water before extraction, $2{\cdot}4\ \mu g$ or 94% of the calculated value was found. No aldosterone ($< 0{\cdot}2\ \mu g$) was found in 53 ml. arterial plasma extracted separately.

In order to determine whether the beneficial effect of adding plasma to cells was simply that of suspending the cells in a larger volume of a watery phase, the experiments on dogs 56 and 66 were carried out. In dog 56 the collected blood was divided into two equal portions. One of these portions

was centrifuged and the cell fraction so obtained again divided into two halves. To the whole-blood portion, to the plasma separated from the cell fraction, and to one portion of the packed cells was each added one volume of water. Three volumes of water were added to the other portion of cells. The results (Table 2) show that suspending the cells in a larger volume of water increased to 78 % of the calculated value the amount of aldosterone that was extracted from the cells. In dog 66 one third of the blood collected was extracted as whole blood. The remaining two thirds were separated into plasma and cells. As before, one volume of water was added to the whole blood sample and to the plasma sample, and three volumes of water were added to the cell fraction. In this instance, the whole of the calculated amount of aldosterone was extracted from the packed cells.

Distribution of corticosteroids between cells and plasma in adrenal venous blood of dogs

Table 3 lists the concentrations of steroids found in plasma and cells of 15 samples of adrenal vein blood from eleven dogs. In some cases the concentration in the cells was calculated by difference from estimates in whole blood and in plasma. Figure 1 illustrates the ratios of concentration in plasma over concentration in cells for the three steroids. The ordinate is on a logarithmic scale. In samples collected for 75–135 min there is usually much more aldosterone in the cells than in the plasma. Cortisol is nearly equally distributed between the two phases. Corticosterone is always found in higher concentrations in the plasma though the excess in plasma is occasionally quite small. It can also be seen that the distribution of the three steroids between plasma and cells varies independently from dog to dog. There is also no consistent relation to the collection time.

Uptake of steroids by the cell fraction

Standing at +4° C

Aldosterone. This experiment was designed to test the time course of the uptake of aldosterone by the cells at +4° C. An adrenal blood sample of a dog was collected into polythene tubes immersed in ice-water. Each tube was placed into the centrifuge and spun exactly 20 min after the start of the collection. Within 2 hr 185 ml. plasma and 195 ml. blood cells had been collected. Two fifths of the plasma and two fifths of the cell sample were extracted immediately. The remaining three fifths of each sample were recombined and kept in the refrigerator (+4° C) for 120 min. The sample was shaken several times. Then it was spun and two thirds of the plasma and two thirds of the cell fraction were extracted separately. The remaining thirds of each fraction were re-combined and extracted as whole blood as a further check on the methods. The results are shown in Table 4.

After short contact between plasma and cells only about one third of the total aldosterone was found in the cell fraction. After a contact of more than 2 hr, over 70% of the aldosterone had attached itself to the cells. There was no loss in the total amount of aldosterone during the time of the experiment.

In addition to the time during which cells and plasma are in contact, there must be other factors which influence the uptake of aldosterone by

TABLE 3. Distribution of steroids (μg/ml.) between plasma and cells in adrenal venous blood of the dog

Dog no.	Collection time (min)	Blood fraction	Aldosterone	Cortisol	Corticosterone
50	70	Plasma	0·009	1·47	0·94
		Cells	0·038	0·74	0·68
51	70	Plasma	0·009	1·52	1·66
		Cells	0·052	2·07	1·40
53	135	Plasma	0·055	1·30	1·13
		Cells	0·038	1·13	0·40
54	90	Plasma	0·019	3·84	2·88
		Cells	0·050	3·74	2·00
$55S_1$	90	Plasma	0·023	2·97	1·46
		Cells	0·035	2·28	1·40
$55S_2$	60	Plasma	0·057	—	—
		Cells	0·083	—	—
56	90	Plasma	0·012	3·61	2·78
		Cells	0·096	4·22	2·08
57	75	Plasma	0·026	3·22	1·84
		Cells	0·027	2·67	1·24
60	75	Plasma	0·026	1·01	0·93
		Cells	0·039	0·84	0·47
$60S_2$	75	Plasma	0·045	1·97	1·59
		Cells	0·090	1·82	0·42
61	75	Plasma	0·038	2·16	1·77
		Cells	0·035	2·25	1·50
$61S_2$	66	Plasma	0·114	5·32	3·78
		Cells	0·024	3·15	1·22
62	75	Plasma	0·057	3·93	2·79
		Cells	0·025	3·35	1·73
64	75	Plasma	0·012	4·00	2·91
		Cells	0·062	5·05	1·26
$64S_2$	75	Plasma	0·025	9·88	3·94
		Cells	0·154	6·58	1·07
Mean concentration		Plasma	0·035	3·30	2·17
		Cells	0·057	2·85	1·21
Mean ratio: $\frac{\text{concentration in plasma}}{\text{concentration in cells}}$			0·61	1·16	1·80

TABLE 4. Migration of aldosterone from plasma to cells in adrenal venous blood of the dog on standing for 2 hr at +4° C

Fraction	Time of contact between plasma and cells (min)	Volume (ml.)	Aldosterone (μg total)	Aldosterone (μg/ml.)	Concn. in plasma / Concn. in cells
Plasma	1–20	74	1·61	0·0218	2·09
Cells	1–20	78	0·81	0·0104	
Plasma	121–140	75	0·67	0·0089	0·40
Cells	121–140	76	1·67	0·0220	
Whole blood	—	76	1·24	—	—

the cells. This can be seen from Fig. 1. Sample 1 of dog 56 and sample 2 of dog 61 were centrifuged after approximately the same time of standing in ice-water. The cells of sample 56_1 contained the largest relative amount of aldosterone, those in sample 61_2 the smallest, ever observed in our experiments.

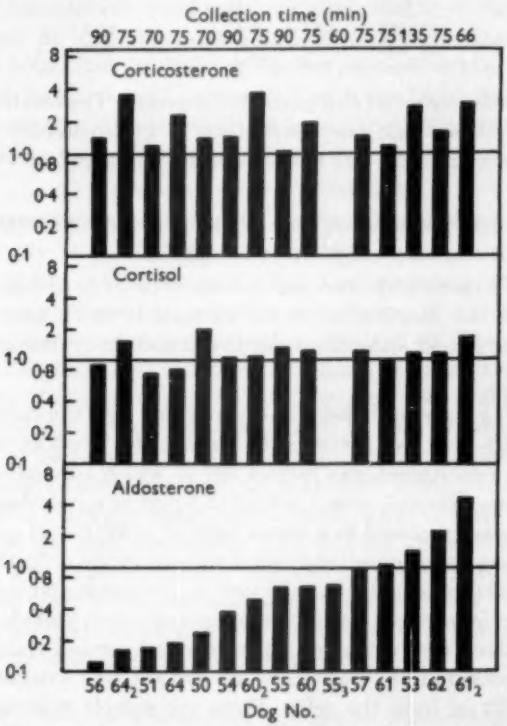

Fig. 1. Distribution of corticosteroids between plasma and cells of adrenal venous blood from the dog. Each bar represents the ratio (concentration in plasma): (concentration in cells) of a single sample. Different samples from the same dog are distinguished by the numeral below the dog number. Logarithmic ordinate scale.

Corticosterone and cortisol. Table 5 illustrates the corresponding experiment on the uptake by blood cells of corticosterone and cortisol. Dog adrenal venous blood was collected for 15 min into an ice-cooled polythene centrifuge tube and spun. Two fifths of the plasma and two fifths of the cell fraction were extracted immediately. The remaining three fifths of each sample were recombined, allowed to stand for 2 hr at +4° C, spun, two thirds of each fraction were extracted separately, and the two

TABLE 5. Cortisol and corticosterone in plasma and cells of adrenal venous blood of the dog before and after standing for 2 hr at +4° C

Fraction	Time of contact between cells and plasma (min)	Volume (ml.)	Cortisol (μg)	Corticosterone (μg)
Plasma	1–15	8·4	24·9	16·5
Cells	1–15	7·0	18·7	9·9
Plasma	121–135	8·4	21·5	15·7
Cells	121–135	7·0	22·5	11·6
Whole blood	—	7·7	20·1	13·2

remaining thirds combined and extracted together. The results are listed in Table 5. They show that the distribution of corticosterone and cortisol between plasma and cells has not significantly changed on standing.

In vivo distribution of cortisol and corticosterone between plasma and cells

Two sets of experiments were carried out in order to obtain information as to whether the distribution of the steroids between cells and plasma observed in the cooled, shed blood samples is also that existing *in vivo*, or is an artifact.

Collection at body temperature. It was possible that the entry of steroids into the cell fraction was favoured by cooling the blood as soon as it left the body. An experiment was carried out in which adrenal venous blood from a dog was collected over a period of 5 min in a polythene centrifuge tube, which was immersed in a water-bath of +37° C and gently shaken. The sample was spun immediately after the end of the collection. A second sample was then collected in a tube kept in ice-water and not shaken. It was also spun immediately after the 5 min collection period. The plasma and cell fractions were extracted separately and cortisol estimated. From the sample collected at +37° C, 24 μg cortisol was extracted from the plasma and 22 μg from the cells. From the sample collected at +2° C, 22 μg cortisol was extracted from the plasma and 23 μg from the cells. The short period of cooling had obviously not modified the distribution.

Collection in the presence of sodium fluoride. It was possible that glycolysis was involved in the association of steroids with blood cells. The inhibition of glycolysis during collection of the blood would then produce results different from those obtained with the usual collection procedure. Two 5-min samples of adrenal venous blood of a dog were collected in the following way. A polythene centrifuge tube containing one drop of a 0·65% solution of NaF was immersed in a water-bath at +37° C and blood collection into it started. Throughout the collection 1 ml. of the NaF solution was added drop by drop to the blood in the tube, which was constantly shaken and then spun immediately. The final concentration of NaF in the

sample was 0·045 %, which is considered sufficient to inhibit glycolysis (Evans, 1922). A second sample was collected in the same way but 1 ml. of 0·9 % NaCl solution was added instead of the NaF solution. Corticosterone and cortisol were estimated in the cells and plasma of both samples. In the sample to which NaF was added 56 % of the total cortisol and 49 % of the total corticosterone were extracted from the cells. In the control sample 51 % of the cortisol and 46 % of the corticosterone were found in the cells. This result lends further support to the view that the association of corticosterone and cortisol with the blood cells takes place *in vivo*, immediately after their release from the adrenal cortical tissue.

Saline washing of blood cells

The stability of the association between cells and steroid was tested in two experiments. 40 ml. of dog's adrenal venous blood which had been standing for 3 hr at +4° C was spun. To a 5 ml. portion of the cells 15 ml. of saline was added and the mixture was shaken gently for 15 min at +21° C. It was then spun and the saline pipetted off. The cells were resuspended in another 15 ml. of saline, shaken for 15 min and spun. Corticosterone and cortisol were estimated in the washed cells and in each saline washing. These steroids were also estimated in 5 ml. untreated cells and 5 ml. plasma of the same blood. The extracting solvents were added to the samples after all of them had been kept at +21° C for a total of 90 min. This was done to allow any losses, attributable to the temperature at which the washing of the cells was carried out, to take place in the unwashed samples too. The experiment was repeated on another sample of dog's adrenal venous blood which had been kept for 6 days at +4° C (experiment 2). The results (Table 6) show that a large amount of the corticosterone and cortisol was washed off the cells by the saline.

TABLE 6. Removal of cortisol and corticosterone attached to cells of adrenal venous blood by washing with saline

Steroids extracted from	Experiment 1			Experiment 2		
	Volume (ml.)	Cortisol (μg)	Corticosterone (μg)	Volume (ml.)	Cortisol (μg)	Corticosterone (μg)
Plasma	5	18·0	8·4	4	12·6	10·2
Cells	5	14·5	6·1	4	10·9	4·8
Washed cells	5	4·8	2·7	4	2·3	0·7
1st saline washing	15	9·3	3·2	12	12·1	4·0

Second saline washings: steroids were also detected in the second saline washings. The amounts were, however, near the threshold of the method and the values are therefore not listed in the table.

Presence of steroids in cells of arterial blood of the dog

Blood was collected from the carotid artery of a dog under ether anaesthesia. It was centrifuged. 250 ml. of the cells was diluted with 750 ml. of water and extracted. The extract was tested for corticosterone, cortisol and aldosterone. 8 μg corticosterone and 12 μg cortisol were found. There was an U.V.-absorbing spot visible in the region of aldosterone diacetate on the third chromatogram, but the eluate of this region did not react with blue tetrazolium. The concentration of cortisol in the cells was of the same order as the average concentration of cortisol in the plasma of blood from the carotid artery: in eight dogs there was a mean of 9·8 μg cortisol in 250 ml. plasma (range: 5·7–12·1 μg).

Distribution of steroids between plasma and cells of adrenal venous blood from rabbit, rat and guinea-pig

Adrenal venous blood samples were taken from a rabbit, a rat and a guinea-pig. The blood was collected into siliconed glass tubes kept in ice-water, spun, and the glucocorticoids were estimated in the plasma and cells. The results are shown in Table 7. In these three species the distribution of the glucocorticoids between plasma and blood cells was similar to that in the dog, about 75 % of the corticosterone being found in the plasma, and the cortisol being distributed equally.

TABLE 7. Distribution of corticosteroids between plasma and cells in adrenal venous blood of rabbit, rat and guinea-pig

Species	Collection time (min)	Fraction	Volume (ml.)	Steroid	(μg total)	(μg/ml.)	Concn. plasma / Concn. cells
Rabbit	20	Plasma	11	Corticosterone	9·0	0·82	3·28
		Cells	10		2·5	0·25	
Rat	35	Plasma	2·5	Corticosterone	17·6	7·04	2·42
		Cells	2·2		6·4	2·91	
Guinea-pig	20	Plasma	2·0	Cortisol	12·6	6·30	0·95
		Cells	2·5		16·6	6·64	

Comparison of values for the secretion rate of corticosteroids, calculated from estimates in plasma and in whole blood

Basic secretion rate in dogs. 'Control' samples of adrenal venous blood were collected from dogs at the beginning of ten experiments, the lost blood being replaced by slow infusion of donor blood. Each sample was divided into two parts. One part was extracted as whole blood, the other centrifuged and the plasma extracted. For each dog the secretion rate of aldosterone, corticosterone and cortisol by the left adrenal was calculated, based on the estimations in plasma and in whole blood and expressed as

μg/adrenal/hr/kg body weight. The mean, the standard deviation of the mean and the coefficient of variation were calculated for each group of figures (Table 8). As was to be expected from the findings on the distribution of aldosterone between plasma and cells, the mean values calculated on the basis of whole-blood estimations are more than three times as large as those calculated from the plasma content only. Furthermore, the coefficient of variation of the secretion rate of aldosterone between individual dogs is smaller when the figures are based on values in whole blood. This agrees with the previous finding that there are large individual variations in the association of aldosterone with blood cells. In contrast, the secretion

TABLE 8. Calculation of secretion rates based on concentrations of steroids (*a*) in plasma and (*b*) in whole blood (μg steroid secreted/hr and kg body weight by the left adrenal)

Dog no.	Collection time (min)	Aldosterone		Cortisol		Corticosterone	
		Plasma	Whole blood	Plasma	Whole blood	Plasma	Whole blood
50	70	0·12	0·68	19·5	32·3	12·4	22·7
51*	70	0·08	0·58	12·1	32·7	13·3	27·1
53	135	0·31	0·57	6·8	13·2	5·9	8·7
54†	90	0·08	0·33	22·8	35·3	12·8	17·0
55	90	0·18	0·51	22·6	44·5	11·1	24·8
56*	90	0·10	0·66	24·7	52·4	21·1	35·3
57	75	0·23	0·50	30·2	53·3	16·3	28·9
60	75	0·31	0·89	10·8	23·0	10·1	16·8
61	75	0·28	0·55	15·8	33·4	12·9	23·5
64	75	0·06	0·38	19·8	46·0	14·5	21·0
Mean		0·175	0·565	18·5	36·6	13·0	22·6
Standard deviation		0·1	0·16	7·1	12·7	3·9	7·3
Coefficient of variation (%)		57	28	38	35	30	32

* Splanchnic nerves cut on left side; † splanchnic nerves cut on both sides.

rate of cortisol and corticosterone, when based on concentrations in blood, is only about twice that based on plasma concentration, reflecting the fact that approximately equal amounts of these steroids are found in plasma and in cells. Individual variations are not greater when plasma is used as the basis for the calculation. This is due to the greater constancy of the distribution of corticosterone and cortisol between plasma and cells.

Estimation of changes in the secretion rate of steroids occurring during the collection of consecutive blood samples from the same dog

Experiments were carried out in order to investigate whether, within an experiment on one dog, changes observed in the secretion rate of adrenal steroids were the same when calculation was based on the steroid content of plasma or that of whole blood. Two samples were collected from each of five dogs. In three dogs the splanchnic nerves were cut before collecting

the second sample, in one dog the second sample was taken 1 hr after a severe haemorrhage, when the adrenal blood flow was very slow. In these four experiments (Table 9) each sample was divided into two portions, one portion was spun and the plasma alone extracted, the other portion was extracted as whole blood. The percentage change in the secretion rate of aldosterone was then calculated from estimates in the plasma fraction only and from estimates in whole blood. In addition, Table 9 contains the percentage of the total amount of aldosterone present in the plasma fraction. In dogs 55, 60 and 64 any alterations in aldosterone secretion occurring in whole blood were reflected in the plasma content. In these dogs the

TABLE 9. Secretion rates of aldosterone during the collection of two consecutive samples, calculated from estimations in plasma and from estimations in whole blood

Dog no.	Sample no.	Treatment between collection of samples	Time of collection (min)	Adrenal blood collected (ml.)	Homologous blood infused (ml.)	Haematocrit (% red cells)	Aldosterone in plasma (content of blood = 100)	% change in secretion rate between samples when estimated in	
55	1	Bled	90	455	450	56	34·7	Blood	−26
	3		60	88	273	56	35·2	Plasma	−25
60	1	Splanchnotomy	75	370	341	57	32·1	Blood	+12
	2		75	210	375	55	29·2	Plasma	+2
61	1	Splanchnotomy	75	360	280	46	50·0	Blood	−15
	2		66	142	600	55	79·5	Plasma	+35
64	1	Splanchnotomy	75	166	150	51	15·9	Blood	+22
	2		75	88	175	47	15·8	Plasma	+21

percentage of the total aldosterone found in plasma was the same in both samples. In dog 61 an interesting discrepancy arose: after splanchnotomy, it was difficult to keep up the blood pressure by infusing donor blood, and 600 ml. of donor blood had to be infused within 1 hr, whilst only 142 ml. blood was collected from the adrenal vein. Aldosterone secretion, calculated on the basis of the concentration in blood, was slightly lower in the second sample. When, however, calculation was based on the values in the plasma, secretion rate appeared to have risen. This was due to the fact that the distribution of aldosterone between plasma and cells had shifted in favour of the plasma during the course of the experiment, a shift probably caused by the infusion of excessive amounts of foreign blood. In Table 3 it was shown that the distribution of aldosterone between plasma and cells is subject to large individual variations, and the infusion of a large volume of blood from another dog may, therefore, lead to a serious alteration in the relative amount of aldosterone in cells and plasma.

In a fifth dog (131, Table 10) the effect on the distribution of steroids between cells and plasma of infusing a large quantity of dextran was tested. 300 ml. of Dextraven (dextran 6 g/100 ml., in glucose solution, 5 g/100 ml.,

salt-free, Benger Laboratories Ltd.) were infused between the collection of sample one and sample two, and another 350 ml. Dextraven during the collection of sample two. Both samples were collected over a period of 45 min into ice-cooled polythene tubes and the samples were centrifuged. Cells and plasma were not in contact with each other for more than 30 min. Before extraction one volume of water was added to the plasma samples and three volumes to the cell samples. When calculation of the secretion rate of aldosterone was based on estimations in plasma, it appeared to have

TABLE 10. Changes in the distribution of corticosteroids between plasma and cells in two 45-min samples of adrenal vein blood (Dog 131). After collection of sample 1 infusion of 300 ml. dextran dissolved in glucose

1	2	3	4	5	6	7		8	9	10	11	12	13
				Concentration in plasma				Secretion rate of left adrenal (μg/hr/kg b.w.)					
				Concentration in cells				Aldosterone		Cortisol		Corticosterone	
Sample no.	Adrenal vein blood collected (ml.)	Haemato-crit (%)	Homologous blood infused (ml.)	Aldosterone	Cortisol	Corticosterone		μg	% change	μg	% change	μg	% change
1	330	64	100	1·19	0·95	1·21	Plasma	0·20	—	17·5	—	12·3	—
							Whole blood*	0·46	—	49·6	—	29·9	—
2	270	37	75, + 350 dextran	0·61	1·61	1·44	Plasma	0·25	+25	27·9	+59	16·1	+31
							Whole blood*	0·49	−0·6	38·1	−23	22·7	−24

* Sum of quantities estimated separately in cells and plasma.

risen in sample two. However, when one adds the amount found in plasma to that found in the cells it is obvious that there was no rise. Further scrutiny shows that it is only by chance that the discrepancy between these two methods of assessment is not greater still. The infusion of dextran brought about two changes which had opposing effects. First, it caused a decrease in the haematocrit of the second sample, which would tend to increase the fraction of the total aldosterone present in the plasma. Second, it caused an increase in the association of aldosterone with the blood cells; this, on its own, would decrease the fraction of the total aldosterone present in the plasma.

The discrepancies were larger still when the changes in the secretion rates of cortisol and corticosterone were calculated from estimates in plasma and compared with those calculated for whole blood. Whereas the dextran caused a shift of the aldosterone to the cells, it altered the distribution of the cortisol in favour of the plasma (column 6, Table 10). That shift, together with the larger plasma volume of sample two, caused a rise in the amount of cortisol in the plasma of sample two. This, however, did not represent a rise in secretion, because there was a very large fall of the cortisol content of the cell fraction. Calculated for whole blood the real change in secretion rate was a fall of 23%.

Plasma estimations of corticosterone also gave a wrong impression of the change in the secretion rate during collection of sample two (last column, Table 10), but the discrepancy was less than for cortisol owing to the smaller change in the distribution coefficient of corticosterone between plasma and cells (column 7, Table 10).

From the results of these experiments it follows that estimates of steroids in two samples of plasma will only reflect changes in the secretion rate of the steroids when the haematocrit and the distribution coefficient of the steroid between plasma and cells remain constant. Both these factors can change during an experiment if large amounts of foreign blood or dextran are infused. Correction of plasma estimates for changes in the haematocrit serves no useful purpose unless it is known that the distribution of the steroid between plasma and cells has remained the same.

DISCUSSION

The experiments have demonstrated the presence of corticosteroids in the cell fraction of adrenal venous blood in four different species. This fact has not been fully appreciated by other workers, possibly because of the difficulty in extracting steroids from the blood cells. There is little doubt that the steroids extracted from the cells are chemically identical with those extracted from the plasma.

The association of steroids with the cells is easily destroyed by washing the cells with saline. This suggests that the cell-bound steroids are available for use by the tissues. The low concentration in the cells of carotid arterial blood supports this view.

We have no evidence on the type of cell with which the steroids are associated. The cell fraction in these experiments consisted of red cells, white cells and some platelets. There is also no conclusive evidence on the site of this association, whether it be intracellular or at the surfaces of the cells, though the easy removal by washing or during circulation in the vessels suggests an attachment to the surface. The volume of plasma trapped between packed cells is too small to account for the large amount of steroids found.

The possibility that the presence of steroids in association with the cells arises as an artifact during the collection of the blood was investigated for cortisol. Quick collection of blood maintained at 37° C and followed by as rapid a separation as possible of the cells from the plasma, did not reduce the amount of cortisol extractable from the cells; this was so whether or not glycolysis was inhibited during collection. It therefore appears that association of glucocorticoids with the cells occurs to a large extent *in vivo*.

On the other hand, experiments carried out on the uptake of aldosterone

when blood was stored at +4° C suggested that *in vivo* less aldosterone is associated with the cells than was found by our method of collecting the blood over a long period of time before separating the cells from the plasma. Whereas during the time taken for collection there is a migration of aldosterone from plasma to the cells, no such shift was observed with cortisol and corticosterone.

The possibility that the cell fraction of the blood acts as a carrier mechanism of hormones *in vivo* has to be seriously considered. Binding to blood cells *in vitro* also occurs with other hormones. Cohen (1959) has reported that more than 70% of adrenaline and noradrenaline added in small quantities to peripheral human blood can be taken up by blood cells. The kinetics of the association of hormones with blood cells are so far unknown.

Previously estimations of the secretory activity of the adrenal cortex were often based on observations made on plasma extracts alone. The experiments described here suggest that using this method of estimation may lead to errors. Secretory changes indicated by estimation of corticoids in plasma are only reliable if the haematocrit and the degree of association of the steroid with the blood cells is the same in both samples. Changes of these two factors were observed after infusion of large amounts of homologous blood or of dextran.

SUMMARY

1. Corticosteroids were extracted from the cells of adrenal venous blood of four mammalian species.

2. In 15 samples of adrenal venous blood of dogs collected over 60–120 min the ratio of concentrations [plasma aldosterone]:[cell aldosterone] varied from 0·125 to 4·7, with a mean of 0·61. Cortisol was found in approximately equal amounts in cells and plasma. The concentration of corticosterone was usually higher, and never lower, in the plasma than in the cells.

3. The steroids could be removed from the cells by washing with saline. Only small amounts of cortisol and corticosterone were found in the cell fraction of blood from the carotid artery of a dog, the concentration being of the same order as that of the plasma. No aldosterone ($< 0{\cdot}2\ \mu g$) was detected in as much as 250 ml. of packed red cells from carotid blood.

4. The proportion of corticosteroids, particularly of aldosterone, associated with the cells in adrenal vein blood is so variable that it is preferable to estimate these steroids in whole blood rather than in plasma when the secretion rate of these hormones is under investigation. Extraction of steroids from red cells presents no difficulties if enough water is added to the packed cells before extracting with the organic solvent.

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REFERENCES

BIBILE, S. W. (1953). The assay of cortical steroids by the mouse eosinophil test. *J. Endocrin.* **9**, 357–369.

BLISS, J. Q., JOHNS, D. G. & BURGEN, A. S. V. (1959). Transfusion reactions due to plasma incompatibility in dogs. *Circulation Res.* **7**, 79–85.

BUSH, I. E. (1952). Paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues. *Biochem. J.* **50**, 370–378.

BUSH, I. E. & SANDBERG, A. A. (1953). Adrenocortical hormones in human plasma. *J. biol. Chem.* **205**, 783–793.

BUSH, I. E. (1957). The physicochemical state of cortisol in blood. *CIBA Foundation Colloquia on Endocrinology*, **11**, 263–285.

COHEN, G. (1959). Storage of adrenaline and noradrenaline by erythrocytes. *Fed. Proc.* **18**, 378.

EBERLEIN, W. R. & BONGIOVANNI, A. M. (1955). New solvent systems for the resolution of corticosteroids by paper chromatography. *Arch. Biochem. Biophys.* **59**, 90–96.

EIK-NES, K., NELSON, D. H. & SAMUELS, L. T. (1953). Determination of 17, 21-hydroxy-corticosteroids in plasma. *J. clin. Endocrin.* **13**, 1280–1282.

EVANS, C. L. (1922). Acid production in shed blood. *J. Physiol.* **56**, 146–156.

HOLZBAUER, M. (1957). The corticosterone content of rat adrenals under different experimental conditions. *J. Physiol.* **139**, 294–305.

HOLZBAUER, M. & VOGT, M. (1957). Functional changes produced in the adrenal cortex of the rat by administration or by release of corticotrophin. *J. Physiol.* **138**, 449–459.

MIGEON, C. J., SANDBERG, A. A., DECKER, H. A., SMITH, D. F., PAUL, A. C. & SAMUELS, L. T. (1956). Metabolism of 4-C^{14}-cortisol in man: body distribution and rates of conjugation. *J. clin. Endocrin.* **16**, 1137–1150.

PETERSON, R. E., WYNGAARDEN, J. B., GUERRA, S. L., BRODIE, B. B. & BUNIM, J. J. (1955). The physiological disposition and metabolic fate of hydrocortisone in man. *J. clin. Invest.* **34**, 1779–1794.

REMINGTON, J. W. & BAKER, C. H. (1959). Plasma volume changes accompanying reactions to infusions of blood or plasma. *Amer. J. Physiol.* **197**, 193–200.

SAFFRAN, M. & SHARMAN, D. F. (1960). An eluter for paper chromatograms. *Canad. J. Biochem. Physiol.* **38**, 303–305.

VOGT, M. (1955). Inhibition by hexoestrol of adrenocortical secretion in the rat. *J. Physiol.* **130**, 601–614.

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THE DISTRIBUTION AND METABOLISM OF THYROXINE AND 3:5:3′-TRIIODOTHYRONINE IN THE RABBIT

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3:5:3′-Triiodo-L-thyronine (T_3) is synthesized and secreted by the thyroid gland of many species. In most assays *in vivo* it has been found to have from five to seven times the biological potency of the major thyroid hormone L-thyroxine, (T_4), (Pitt-Rivers & Tata, 1959). Certain exceptions have been observed, however. In the chicken, Gilliland & Strudwick (1953), studying the inhibition of ^{131}I release from the thyroid, Shellabarger (1955) using the goitre prevention assay, and Newcomer (1957) studying O_2 consumption and feather growth, found the two compounds to be nearly equipotent. Brown-Grant (1955) showed that T_3 and T_4 had the same activity in suppressing thyrotrophic hormone (TSH) secretion in the rabbit as judged by inhibition of the release of ^{131}I-labelled hormone from the thyroid gland.

Until recently no satisfactory explanation was available for these differences. The recognition of the role played by specific thyroxine-binding proteins of the plasma in determining the biological half-life and the rate of entry of thyroid hormones into the tissues is a fairly recent development (Pitt-Rivers & Tata, 1959; Tata, 1960*a*; Brown-Grant, 1960). It has been demonstrated that in the chicken no specific thyroxine-binding globulin (TBG) is present in the plasma. Consequently, the affinity for and the capacity to bind T_4 is much lower in chicken than in human plasma. The major binding protein of chicken plasma is found in the albumin fraction and has identical affinities for T_3 and T_4 (Tata & Shellabarger, 1959). Thus both hormones would be expected to have the same short half-life (a value of 22·5 hr was in fact observed) and to reach the tissues at similar rates. This could explain the similarity of the biological activities in this species.

It seemed possible that a similar situation might exist in the rabbit, and the distribution and metabolism of ^{131}I-labelled T_3 and T_4 in the rabbit and the binding to serum proteins *in vivo* and *in vitro* have therefore been studied.

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METHODS

Young adult rabbits of mixed breeds (1·5–2·0 kg body weight) were used. Animals of the same sex from two litters were studied in each series of experiments and the groups were chosen so that the mean body weights were approximately the same and that equal numbers of animals from each litter received T_3 and T_4. The animals were kept in a light- and temperature-controlled (18–20° C) animal room for at least 2 weeks before and during the course of the experiments and maintained on pellets (Diet 18, Bruce & Parkes, 1947) and tap water *ad lib.* In some experiments a solution of KI in tap water (0·002 g/100 ml.) was given instead of tap water, as described under Results.

The hormones labelled with ^{131}I in the phenolic ring were obtained from Abbot Laboratories Ltd, and were shown by chromatography to be at least 85% pure. The stock solutions (in 1:2-propane-diol) were diluted with a 1:9 (v/v) mixture of rabbit plasma and NaCl solution 0·9 g/100 ml. before use and were injected intravenously in a volume of 0·5–1·0 ml. Labelled iodinated human serum albumin was obtained from the Radiochemical Centre, Amersham, England, and was diluted with 0·9% NaCl solution before injection. In each experiment an internal standard was prepared from the diluted stock solution under the same injection conditions.

Blood was drawn from the marginal vein of the ear and clotting prevented by adding a small amount of heparin powder. Plasma was obtained by centrifugation and a known volume was diluted to 10 ml. and counted to determine total plasma radioiodine content. The protein-bound radioactivity (PB ^{131}I) was determined by precipitating the plasma proteins with 5 volumes of trichloracetic acid (TCA) solution, 10 g/100 ml., resuspending after centrifuging, washing twice with 10% TCA and finally dissolving the precipitated protein in 3N-NaOH to a volume of 10 ml. and counting this solution. More than 90% of the activity appeared in the PB ^{131}I fraction when T_3 and T_4 were added to rabbit plasma *in vitro* and less than 5% when radioiodide was added *in vitro*. Tissue samples weighed after washing and gently blotting dry were also dissolved in 3N-NaOH for assay of their ^{131}I content. All counts were made with an M6 liquid counter to a statistical accuracy of ±2·5% or better. After correction for isotope decay, the results were expressed as a percentage of the administered dose by reference to the internal standard prepared at the time of injection.

The interaction of thyroid hormones with serum proteins was studied by paper electrophoresis (5 V/cm for 20 hr) in 0·05M veronal buffer, pH 8·6 (Block, Durrum & Zweig, 1955), as described elsewhere (Tata & Shellabarger, 1959). The capacity and relative affinity with which thyroxine and triiodothyronine are bound to the different binding fractions were measured (1) by paper electrophoresis at increasing concentration of added hormones and (2) by the method of displacement of radioactive thyroxine with stable triiodothyronine, as described by Robbins & Rall (1957). As was shown by Myant & Osorio (1959), rabbit serum albumin and α-globulin-type thyroxine-binding globulin cannot be well separated and the error involved in such studies is of the order of ±5–10%.

RESULTS

The distribution of T_3 and T_4 in vivo

In the first experiment eight female rabbits were used: four received T_3 (20 μc, 1·6 μg) and four received T_4 (20 μc, 2·0 μg) intravenously. Blood samples were taken at 30 min and at 6, 25, 49, 72 and 96 hr after injection. Total plasma radioactivity and PB ^{131}I were determined in the early samples. At 49 hr, PB ^{131}I was more than 95% of the total activity and only total activity was determined at 72 and 96 hr. The results are

shown in Fig. 1. As early as 30 min after injection the plasma level of T_3 was much lower than that of T_4; this difference was maintained, but from 25 to 96 hr the fractional rate of disappearance from the plasma for both compounds appeared to be the same. The biological half-life in the blood was estimated to be 27 hr.

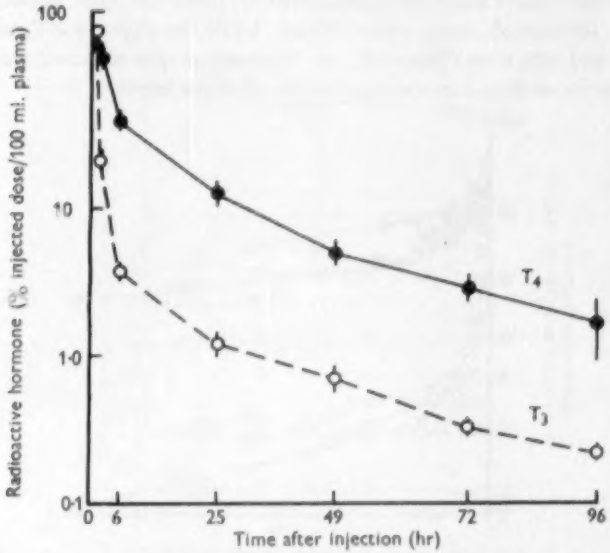

Fig. 1. The concentration of radioactive hormone in the plasma after the intravenous injection of 1·6 μg of T_3 or 2·0 μg of T_4 in rabbits. Each point is the mean value from 4 animals; the vertical bars indicate the standard errors of the means. Between 6 and 96 hr the curves are parallel, indicating similar fractional removal rates. The estimated half-life is 27 hr for both substances. Initial values at 0 hr were calculated on the basis of a complete distribution within the estimated plasma volume (Armin *et al.* 1952).

In these experiments the animals were given an 0·002 % solution of KI, beginning 2 days before and continuing throughout the period of the experiment, in order to prevent the recirculation of ^{131}I from degraded thyroid hormone. For thyroxine the extent of recirculation had previously been estimated to be only 8 % in the rabbit (Brown-Grant & Gibson, 1955). Thus the curve representing results obtained in this earlier study, in which 1 μg of labelled T_4 was injected and whole blood radioactivity was measured, was quite similar to the present results and gave a value of 25 hr for the half-life: in these earlier experiments the ^{131}I content of the thyroid gland 96 hr after injection was 5–7 % of the injected dose, whereas in the present series thyroid ^{131}I at 96 hr was 0·14, 0·26, 0·18 and 0·32 %

(mean 0·23 %) in the case of T_4 and 0·54, 0·27, 0·34 and 0·14 % (mean 0·32 %) in the case of T_3.

The fall in plasma radioactivity after the injection of T_3 was very rapid. Calculations based on the data of Armin, Grant, Pels & Reeve (1952) gave a mean value of 83 ml. for the plasma volume of the animals used in these experiments. The volume of distribution of T_4 30 min after injection was 114, 129, 109 and 89, mean value 110 ml., while the values for T_3 were 884, 951, 951 and 560, mean value 837 ml. The early stages of distribution were studied in more detail in a second series of experiments.

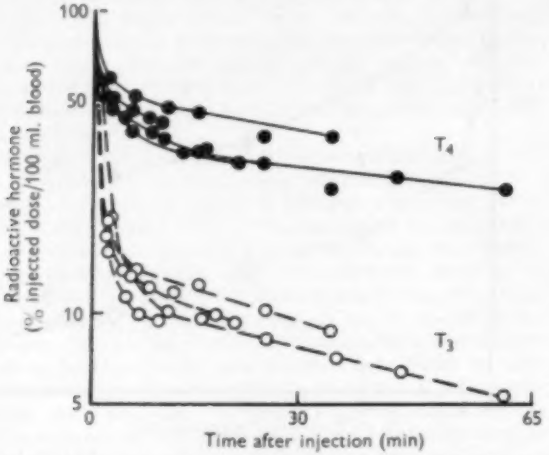

Fig. 2. The concentration of radioactivity in whole blood after the intravenous injection of radioactive T_3 or T_4 in rabbits. Each curve represents data from an individual animal. Initial point at 0 min is the calculated concentration based on a complete distribution in the estimated blood volume (Armin *et al.* 1952).

Eight female animals were used: four received T_3 (8·8 μc, 1·4 μg) and four received T_4 (6·3 μc, 1·2 μg) intravenously. Blood was collected directly into graduated tubes 1–2 min after injection and at 5–10 min intervals after this. One animal in each series was killed 10, 20, 40 and 65 min after the injection of labelled hormone by means of an overdose of intravenous sodium pentobarbitone and tissue samples were removed for the determination of ^{131}I content. The body fluids and tissues studied were whole blood, plasma, bladder urine, bile, liver, spleen, kidney, skin, muscle and thyroid. The skin sample was the tip of the ear which had not received the initial injection of hormone and the muscle sample was from the lower third of the left quadriceps femoris. Only portions of the liver and kidney were assayed, but the total weight of these organs was recorded.

The fall in blood radioactivity is shown in Fig. 2; the difference between

T_3 and T_4 is well established by 5–10 min after injection. Yet although the blood levels of the two groups are so different there is no evidence of a major difference in the total amount of hormone present in the other tissues examined, when expressed as percentage of the administered dose (Fig. 3), or of any consistent difference in the concentration (% dose/g tissue) (Table 1). The ratio of tissue to plasma ^{131}I concentration (T/P

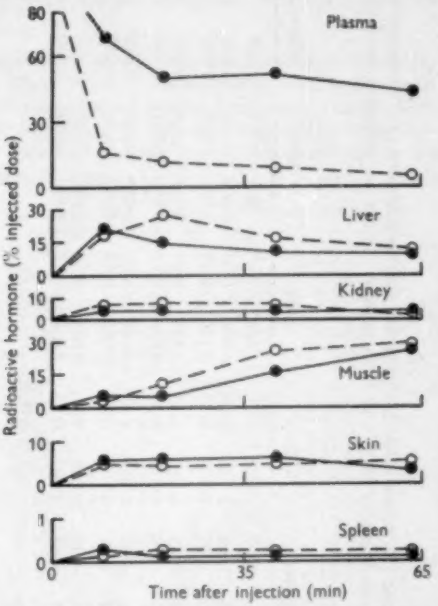

Fig. 3. The percentage of the administered dose of ^{131}I-labelled hormone found in various organs after the intravenous injection of a tracer dose of T_3 and T_4 in rabbits. The muscle mass was estimated as 50% of body weight and skin as 5% of body weight. These are values obtained before correction for hormone in the vascular compartment of each organ. ●——● thyroxine (T_4); ○- - -○ triiodothyronine (T_3).

ratio) is consistently higher for T_3 in all organs showing a significant ^{131}I content and in bile at each of the four time intervals studied (Table 1). The chemical nature of the ^{131}I in the tissues was not determined.

In view of the marked differences in the plasma concentrations of T_3 and T_4 even at these short periods after injection, considerable differences in the ^{131}I content of the organs examined were expected but not observed. The possibility that T_3 might be concentrated in some organ or tissue other than those examined in these first experiments was investigated in male rabbits. Four rabbits were injected with T_3 (6 μc, 0·6 μg) and four

TABLE 1. The concentration of labelled hormone (% injected dose/g tissue) and the tissue/plasma (T/P) concentration ratio for selected organs at varying intervals after the intravenous injection of T_3 and T_4 in rabbits. Values not corrected for hormone in the vascular compartment

Hormone	Time after injection (min)	Liver		Spleen		Kidney		Skin		Muscle		Thyroid		Bile	
		%/g	T/P	%/g	T/P	%/g	T/P	%/g	T/P	%/g	T/P	%/g	T/P	%/g	T/P
T_3	10	0·302	1·43	0·076	0·36	0·420	1·98	0·056	0·26	0·004	0·02	0·040	0·20	0·240	1·13
T_4	10	0·258	0·26	0·370	0·37	0·358	0·36	0·149	0·15	0·058	0·06	0·060	0·06	0·040	0·04
T_3	20	0·331	2·37	0·113	0·64	0·562	4·02	0·052	0·37	0·015	0·10	0·094	0·67	0·193	1·37
T_4	20	0·207	0·32	0·057	0·09	0·430	0·66	0·071	0·11	0·008	0·01	0·084	0·01	0·062	0·95
T_3	40	0·370	2·54	0·175	1·26	0·618	4·43	0·060	0·39	0·036	0·26	0·240	1·71	0·140	1·01
T_4	40	0·225	0·29	0·083	0·11	0·590	0·77	0·096	0·13	0·013	0·02	0·186	0·21	0·068	0·09
T_3	65	0·180	2·47	0·105	1·44	0·352	4·77	0·054	0·74	0·024	0·33	0·168	2·18	0·053	0·72
T_4	65	0·189	0·44	0·067	0·16	0·257	0·59	0·052	0·12	0·057	0·13	0·200	0·40	0·400	0·91

with T_4 (5 μc, 0·5 μg) and killed 30 min later. In addition to the organs examined in the previous study, the ^{131}I content of the adrenal gland, the heart, the genital tract, stomach, small intestine, large intestine, lungs, diaphragm, tongue, salivary glands, pancreas and thymus was determined. In addition, multiple samples of liver, skin and muscle rather than single samples were assayed. None of the organs examined had a strikingly higher content of ^{131}I after the injection of T_3 as compared with the content

TABLE 2. Distribution of ^{131}I 30 min after the intravenous injection of labelled T_3, T_4 and iodinated albumin. Each value (% administered dose) is the mean of results from four rabbits. Total muscle content calculated on the basis of a muscle mass equal to 50% of the body weight and blood content on the estimated blood volume from the data of Armin *et al.* (1952). Values for albumin are % dose in each organ and the calculated value for the volume of plasma in each organ

	Direct		Corrected		Iodinated albumin	
Organ	T_3	T_4	T_3	T_4	(% dose)	(ml. plasma)
Adrenal	0·049	0·020	0·047	0·010	0·180	0·113
Bile	0·170	0·150	0·168	0·139	0·027	0·013
Genital tract	0·328	0·441	0·285	0·226	0·388	0·244
Heart	0·572	0·523	0·567	0·317	0·443	0·278
Kidneys	5·885	6·295	5·56	4·65	2·92	1·83
Large intestine	8·83	9·49	8·43	6·285	3·92	2·45
Liver	23·4	18·1	22·7	12·1	10·675	6·69
Lungs	1·18	1·05	0·961	0·280	1·19	0·752
Muscle	27·1	26·4	25·4	15·7	15·75	9·895
Skin	6·01	7·19	5·645	4·33	3·30	2·07
Small intestine	9·51	9·18	7·17	4·32	3·025	1·91
Spleen	0·134	0·110	0·130	0·056	0·130	0·082
Stomach	5·37	5·01	4·57	2·74	3·255	2·04
Salivary glands	0·319	0·237	0·309	0·180	0·094	0·059
Thymus	0·048	0·035	0·046	0·026	0·105	0·066
Thyroid	0·102	0·263	0·090	0·203	0·002	0·001
Blood	14·2	53·5	14·2	53·5	—	—
Total	103	138	96·3	105	—	—

after the injection of labelled T_4 (Table 2). No evidence was obtained to support the hypothesis that the T_3 that had left the vascular compartment of the body was concentrated preferentially in any organ of the body.

The measurements of the ^{131}I content in a wider range of tissues enabled a more complete 'accounting' of the injected radioactivity to be carried out. As shown in Table 2, when the total % recoveries for T_3 and T_4 were calculated on the assumption that muscle accounts for 50% of the body weight (Gross, Ford, Symchowicz & Horton, 1957), and on the basis of the plasma volumes given by Armin *et al.* (1952), the values obtained were 103 and 138% respectively of the injected dose. Errors in the estimated muscle mass or plasma volume could not account for such a discrepancy. It seemed likely that the error arose from a duplication in the measurement of the radioactivity in the vascular compartment of the body, due to the

inclusion of ^{131}I in the blood in the total hormone content of the different organs measured.

The possibilities of repeating the experiments under anaesthesia and terminating them by exsanguinating the animals or of perfusing the animals immediately after death were considered but rejected on the grounds that either procedure would almost certainly lead to gross changes in the distribution of the labelled hormone. Instead, the fraction of the plasma volume contained in each organ as prepared for assay in these experiments was determined from a study of the distribution of the radioactivity after the injection of a small dose of radio-iodinated human serum albumin. The dose injected was 20 μc (830 μg of protein) in a volume of 0·25 ml. The animals were killed 30 min later. From the plasma level of radioactivity, plasma volumes of 29, 33, 35 and 33 ml./kg body weight were calculated. The ^{131}I content of each organ, as a percentage of the injected dose, was determined and the plasma content (^{131}I content of organ divided by ^{131}I content of 1 ml. plasma) was calculated. The mean results are given in Table 2. From the calculated plasma content of each organ, and the known plasma level of radioactivity in each animal that received labelled hormone, it was possible to calculate a corrected organ content of labelled hormone for each animal representing the percentage of the injected dose present after allowing for the amount contained in the vascular compartment. When this was done, it was clear that the tissue content of T_3 was consistently higher than that of T_4 (Table 2), the difference being observed in all organs examined except the thyroid. When the corrected organ contents plus blood content were added together, an average value of 96 % of the T_3 and 105 % of the T_4 injected was accounted for.

At these physiological levels there is no indication that the early stages in the distribution of T_3 and T_4 in the rabbit differ from those observed in the rat or man (Pitt-Rivers & Tata, 1959). The behaviour of the two hormones is consistent with the hypothesis that a specific binding protein(s) with a higher affinity for T_4 than for T_3 exists in the blood of rabbits as of other mammals. This question was examined directly.

Interaction of thyroid hormones with rabbit serum proteins

By paper electrophoresis in the presence of veronal buffer (Tata & Shellabarger, 1959) both thyroxine and triiodothyronine were found to be bound to two major rabbit serum protein fractions: (*a*) an α-globulin (localized between α_1-globulin and albumin) thyroxine-binding globulin (TBG), and (*b*) albumin. In Table 3 it is seen that these two fractions accounted for over 90 % of protein-bound hormonal ^{131}I, whether the hormones were endogenously labelled (36 hr after injection of radioiodide)

or injected or mixed *in vitro*. In a separate experiment it was found that endogenously labelled thyroid hormone consisted of about 90–95% of organic ^{131}I as thyroxine and about 2–6% as triiodothyronine, between 24 and 110 hr after administration of ^{131}I-iodide to a normal rabbit.

Despite the qualitative similarity of thyroid hormone binding, there were differences at the quantitative level when thyroxine-binding in rabbit serum was compared to that in sera of other mammals. The capacity

TABLE 3. Distribution of radioactivity in TBG and albumin of rabbit serum in the presence of ^{131}I-labelled thyroxine (T_4) and triiodothyronine (T_3) under different conditions. The proteins were separated by paper electrophoresis in veronal buffer at pH 8·6

Experimental condition	Labelled hormone present	% ^{131}I distributed in TBG	% ^{131}I distributed in Albumin
Endogenously labelled*	T_4 T_3	74	21
30 min after injection†	T_4	71	23
	T_3	53	40
Added *in vitro*‡	T_4	68	26
	T_3	46	48

* Serum of rabbit, 48 hr after injection of 50 μc of carrier-free ^{131}I-iodide. † As in Fig. 1. ‡ (0·01 μg T_4 and T_3)/ml. serum.

TABLE 4. Capacity of rabbit serum TBG to bind radio-active thyroxine and triiodothyronine as determined by paper electrophoresis in veronal buffer at pH 8·6

Substance added	Amount (μg/ml.)	% ^{131}I distributed in TBG	Albumin	Other*
L-Thyroxine	0·01	68	26	6
	0·05	52	41	7
	0·20	29	60	11
	0·50	10	76	15
3:5:3′-triiodo-L-thyronine	0·01	46	48	6
	0·05	23	65	12
	0·20	7	74	19
	0·50	5	78	17

* Includes β- or γ-globulins or free hormone.

of rabbit serum TBG to bind thyroxine and triiodothyronine is low (Table 4). Assuming that 90% of the average rabbit protein-bound iodine (PBI) value of 5·0 μg/100 ml. plasma (Myant, 1958) is thyroxine, it will be seen that doubling of thyroxine concentration causes a significant degree of saturation of serum TBG. Under similar conditions hardly any radio-active thyroxine would be displaced to the albumin zone of human serum (Robbins & Rall, 1957; Tata, 1960*a*). With triiodothyronine the saturation of TBG was achieved more readily. On the other hand, the capacity (as distinct from 'affinity') of rabbit serum albumin was extremely high; a significant displacement of ^{131}I from this protein (to other protein fractions)

could only be detected when a level of 5μg thyroxine/ml. was exceeded. In this respect it is similar to human and rat serum albumin examined in veronal buffer.

The relative binding affinities for thyroxine and triiodothyronine, determined by the progressive displacement as labelled T_4 by the addition of stable T_3 (Rabbins & Rall 1957) vary with the amount of radioactive thyroxine present (Table 5). At low concentrations of added radioactive T_4 (0·01 μg/ml.), when most of it was bound to TBG (see Table 4), triiodothyronine was bound about three times less firmly in rabbit serum.

TABLE 5. Relative affinities of rabbit serum TBG (whole serum) and serum albumin for thyroxine and triiodothyronine as determined by the method of Robbins & Rall (1957); compared on the basis of thyroxine = 100

Sample	Amount of radioactive thyroxine present (μg/ml.)	Relative affinity for triiodothyronine
Whole serum	0·01	40
	0·05	71
	0·50	82
Rabbit serum albumin	0·01	88
	0·05	95
	0·50	90

However, when these measurements were made at higher concentrations, when more T_4 was bound to the albumin (see Table 4), the relative affinities approached the same value. That rabbit serum albumin binds the two hormones with almost the same affinity is seen in Table 5 from experiments performed on isolated rabbit serum albumin.

The metabolism and excretion of T_3 and T_4

The rapid loss of T_3 from the vascular compartment of the body during the first 6 hr after injection and the finding of a higher general tissue uptake of T_3 than of T_4 are consistent with the observations made on the protein-binding of the two hormones in the serum. However, the observation that from 24 hr after injection to 96 hr the half-lives in the blood are essentially the same, although the levels are very different, raises the question as to the relative half-lives in the body as a whole. As a whole-body γ-counting apparatus for animals of this size was not available, the rate of excretion of ^{131}I in the urine and faeces after the injection of labelled hormone was determined, and the amount remaining in the body calculated and plotted on a semi-logarithmic scale. The animals were kept in individual metabolism cages and given 0·002% KI solution to drink, beginning 48 hr before the injection of hormone and continuing throughout the experi-

mental period. Urine was collected at 8 hr after injection and urine and faeces at 24 hr and then daily.

Four rabbits were injected intravenously with 6·6 μc (0·4 μg) of T_3 and four with 5·0 μc (0·2 μg) of T_4. The percentage of the dose retained in the

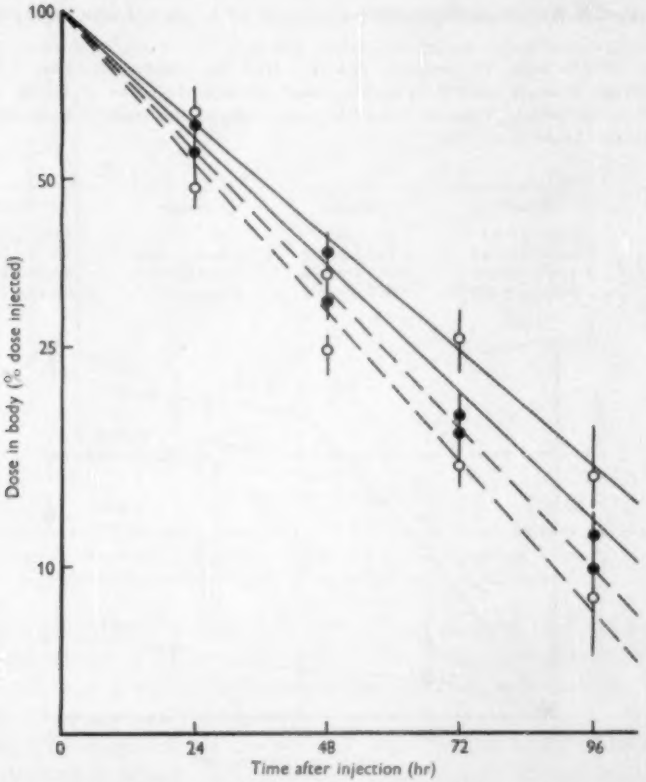

Fig. 4. The % retention of ^{131}I in the body after the administration of a tracer (0·5 μg) or a carrier dose (50 μg) of labelled T_3 and T_4. Each point is the mean of values from 4 rabbits; vertical bars indicate standard errors of the means. ○- - -○ tracer T_3; ○——○ carrier T_3; ●- - -● tracer T_4; ●——● carrier T_4.

body at different times is shown in Fig. 4, and the pattern of urinary and faecal excretion is shown in Figs. 5 and 6. The estimated half-lives for T_3 and T_4 are 26 and 27 hr respectively. In both instances more of the radioactivity appears in the urine than in the faeces. The level of ^{131}I in whole-blood samples is shown in Table 6.

The experiment was repeated with a larger dose of hormone (50 μg) in

order to study the excretion of an excess of T_3 and T_4. The retention of activity within the body is shown in Fig. 4. The mean half-lives, determined graphically, were 34 hr for T_3 and 29 hr for T_4, and thus a slight difference between the mean rate of excretion of T_3 and T_4 in physiological doses, or when given in excess, was observed. The pattern of excretion, however, differed markedly when an excess of hormone was injected. The

TABLE 6. Whole blood ^{131}I levels (% dose/ml.) after the injection of tracer (0·5 μg) or carrier (50 μg) doses of labelled T_3 and T_4. Each value is the mean ± S.E. of mean or results from four rabbits. Value at 0 hr is based on a complete distribution in the estimated blood volume (Armin *et al.* 1952)

Time (hr)	T_3		T_4	
	Tracer	Carrier	Tracer	Carrier
0	1·20 ±0·13	1·08 ±0·17	1·29 ±0·21	1·09 ±0·14
2	0·068±0·010	0·102±0·002	0·343±0·024	0·334±0·039
24	0·012±0·001	0·031±0·008	0·078±0·001	0·093±0·005
48	0·004±0·001	0·009±0·002	0·034±0·001	0·042±0·003

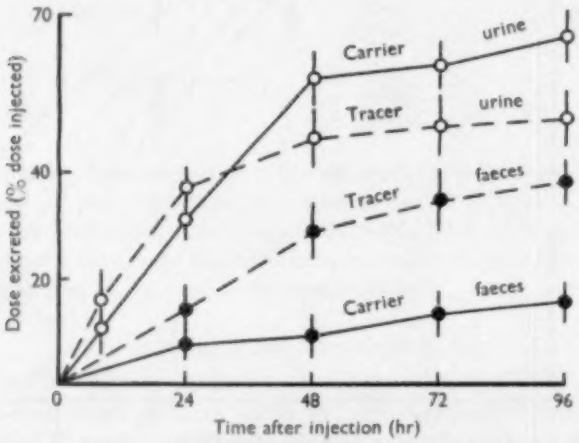

Fig. 5. The cumulative excretion of ^{131}I in urine and faeces after the intravenous injection of a tracer or a carrier dose of labelled T_3. Each point is the mean of values from 4 rabbits; vertical bars indicate the standard errors of the means.

proportion of the activity lost in the urine (as iodide) was increased with respect to the faecal excretion (Figs. 5 and 6). The excretions for the initial periods of 8 and 24 hr were somewhat lower in both cases, but the final urinary excretion was significantly higher. This delay in excretion is of interest with respect to the slightly longer half-lives observed when an excess of hormone is injected and the raised level of ^{131}I in whole blood samples (Table 6).

Two further metabolic experiments were carried out, the results of which are relevant to the discussion of the previous results. The excretion of iodide was studied to determine whether the iodide solution had effectively blocked the uptake of iodide by the thyroid gland and whether there was any significant contamination of the faeces by urinary radioiodide. Four rabbits received 7·5 μc of carrier-free ^{131}I intravenously. Urinary excretion was rapid and complete; the mean cumulative excretion

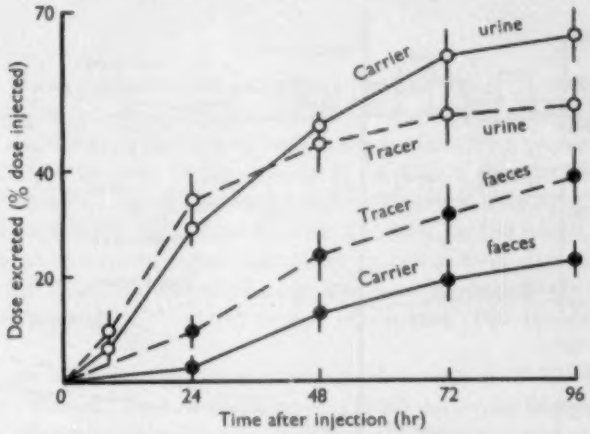

Fig. 6. The cumulative excretion of ^{131}I in urine and faeces after the intravenous injection of a tracer or a carrier dose of labelled T_4. Each point is the mean value from 4 rabbits; vertical bars indicate the standard errors of the means.

was 93·2 ± 1·9, 99·3 ± 0·9 and 100·1 ± 1·0 % (S.E. of mean of 4 determinations) respectively at 24, 48 and 72 hr after injection. Only 0·6 % of the radioactivity was found in the faeces in the first 24 hr and less than 0·2 % in the 24–48 hr period after injection.

During these experiments the animals were kept in metabolism cages that allowed them to move about and to continue their normal behaviour as regards refection. Only hard faecal pellets were obtained in the daily collection. The possibility that the administration of an excess of thyroid hormone might lead to increased refection, with a greater degree of enterohepatic recirculation of hormone and a higher probability of de-iodination rather than faecal excretion as the eventual means of disposal from the body, had to be considered. It seemed likely that any form of restraining device would prove to be a considerable 'stress' to the animals and that this might itself alter the rate or pattern of thyroid hormone metabolism. An indirect method was therefore preferred.

Seven rabbits were kept in metabolism cages for 3 days before the

experiments began. They were offered a known weight of pellets in excess of their daily intake in non-spill hoppers and their daily intake measured to ± 2 g. The faecal material was collected daily and weighed to ± 2 g. A known volume of iodide solution in excess of their daily intake was available and the fluid intake and urine volume were measured daily to within 5 ml. The animals were also weighed daily to ± 10 g. The pattern of solid and fluid intake and output and of weight gain were followed for

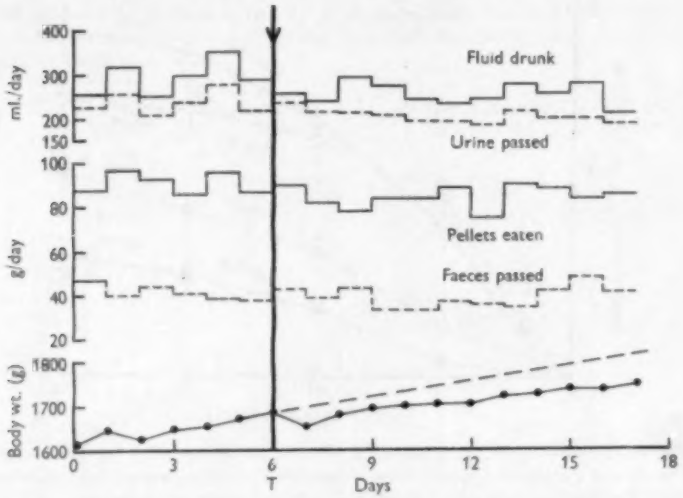

Fig. 7. The effect of 50 μg T_4 intravenously on fluid intake, urine output, food intake, faecal output and on body weight. Each value given is the mean result from 7 rabbits. Note the slight decrease in food intake without significant decrease in faecal mass after the injection of 50 μg T_4 intravenously at T.

6 days. Each animal was then injected intravenously with 50 μg of L-thyroxine in a volume of 0·25 ml. and the study continued for a further 11 days.

Figure 7 shows the results obtained. Following the injection of 50 μg of T_4 there was no obvious change in fluid intake or in urine volume; the excess of fluid drunk over urine collected remained the same. Mean food intake was slightly, but not significantly ($P > 0{\cdot}05$) below the pre-injection level for 6 days after injection (before, 91 ± 2 g/day (S.E. of mean of 42 measurements); after, 87 ± 3 g/day). The faecal mass was not significantly changed (40 ± 2 compared to 37 ± 2 g/day) despite the slight decrease in food intake, which does not suggest that any change in the direction of increased refection had occurred. The steady increase in body weight (pre-injection average 12 g/day) ceased for 3 days but was then

resumed, although the predicted weight was not regained within the 11-days post-injection period of the experiment. There was no support for the hypothesis that a greater degree of enterohepatic recirculation due either to increased refection or associated with a more efficient absorption of intestinal contents resulted from the injection of a large dose of thyroid hormone and could be responsible for the greater proportion of hormone deiodinated under these conditions.

DISCUSSION

In the present experiments on rabbits the half-life of T_3 and T_4 in the blood after the initial rapid fall in blood level during the phase of distribution was studied. The half-life of both compounds after a tracer dose was estimated from the data of Fig. 1 to be 27 hr. This is close to the value of 25 hr for T_4 obtained previously by Brown-Grant & Gibson (1955). The finding of indistinguishable half-lives for T_3 and T_4 in the rabbit is in contrast to observations in other mammals. Values of 6–11 days for T_4 and 2–3 days for T_3 in man and of 16–19 hr and 4–10 hr respectively in the rat have been reported by several groups of workers (Pitt-Rivers & Tata, 1959).

These are species in which the biological potencies of T_3 and T_4 are strikingly different. Tata & Shellabarger (1959), however, found identical half-lives (22·5 hr) in the chicken, in which the hormones are equipotent. Pipes, Premachandra & Turner (1959) obtained values of 1·99 and 2·47 days respectively for T_3 and T_4 in dairy cows, and it had been shown previously that the effects of T_3 and T_4 on the milk yield of cows in declining lactation was very similar (Bartlett, Burt, Folley & Rowlands, 1954). In the rabbit, too, T_3 and T_4 are about equipotent in suppressing pituitary thyrotrophic hormone secretion (Brown-Grant, 1955).

Tata & Shellabarger (1959) have suggested that these two phenomena may be connected with one another and be related, in birds, to the absence of a specific TBP from the serum. This was not found to be the case in the rabbit. Studies on the binding of thyroid hormones to the serum proteins of the rabbit during electrophoresis in barbiturate buffer showed that qualitatively the pattern of binding as well as the demonstrably higher affinity of the TBP for T_4 than for T_3 were as in other mammals and different from the bindings in birds (Robbins & Rall, 1957; Tata & Shellabarger, 1959; Tata, 1960*a*).

The results from studies of the distribution in the tissues of physiological doses of T_3 and T_4 during the first hour after injection are consistent with these observations on serum protein binding. The injected T_3 leaves the blood very rapidly as compared with T_4 (Fig. 2). When organ contents

are compared, after correction for the hormone contained in the vascular compartment, it is clear (Table 2) that the T_3 content of each of the wide variety of tissues examined is higher than that of T_4. However, there is no evidence that during the first hour after injection the pattern of distribution of T_3 and T_4 between organs is grossly different (Fig. 8). In particular, the liver content of T_3 as compared with T_4 is not grossly different from the

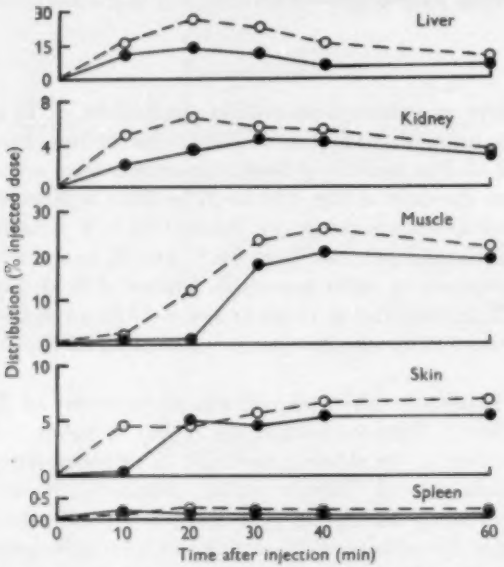

Fig. 8. The distribution of T_3 and T_4 in rabbit tissues 10–65 min after the intravenous injection of a tracer dose. The values given are % of the administered dose after correction for hormone in the vascular compartment, as explained in the text. Except for the values at 30 min, which are mean of results in 4 animals, each point is based on results from a single animal. Note that the general pattern of distribution is the same for both T_3 and T_4, though the values for T_3 are consistently higher. ○---○ T_3; ●——● T_4.

relative T_3 and T_4 contents of, say, the adrenal or the salivary gland or of muscle (Table 2). There is no evidence to support the suggestion of Gross *et al.* (1957) that the passage of T_3 through the liver or kidney and the formation of some conjugate other than the glucuronide is a necessary preliminary to the entry of T_3 into the other tissues of the body. Gross's compound X (the unidentified T_3 conjugate) was not detected on our chromatograms of plasma obtained after T_3 injection. Further, significant tissue levels of ^{131}I have been observed in animals injected with labelled T_4 at a time when no labelled T_3 was found on chromatograms of plasma.

The method used for the determination of the relative biological activities of T_3 and T_4 in the rabbit (Brown-Grant, 1955) depended on the maintenance of a raised level of hormone in the blood. When the level of hormone was raised in the electrophoresis experiments, it was seen that rabbit TBG has a very limited binding capacity for T_4. Table 4 illustrates the saturation of TBP and the displacement to albumin. Even with endogenously labelled hormone at normal plasma levels, a significant proportion of the activity was localized in the albumin zone (Table 3). These findings suggest that rabbit blood contains very little TBG compared with other mammals. The high values for thyroxine-binding capacity reported by Myant & Osorio (1959) may be due to a failure to achieve a comparable degree of separation of albumin and TBP in the two groups of experiments.

Accepting that the binding capacity of rabbit TBG is low, even a relatively small increase in serum hormone level would result in a large fraction of the hormone being bound to serum albumin, which has roughly equal but lower affinities for both T_3 and T_4. Under these circumstances the rate of disappearance of T_4 from the blood might be greatly increased, perhaps even approaching that of T_3 and the half-lives of both compounds might be much shorter. The findings of Balfour & Tunnicliffe (1960) that during electrophoresis in buffer other than barbiturate a significant proportion of the T_4, even at physiological plasma levels, travels with the pre-albumin fraction may necessitate a reconsideration of this hypothesis. The T_3-binding capacity of rabbit pre-albumin is not known, but it has been established that human thyroxine-binding pre-albumin (TBPA) does not bind T_3 at all (Ingbar, 1958; J. R. Tata, X. Y. Widnell & Y. Z. Grazner, unpublished).

The possibility that differences in the firmness of binding to serum proteins at raised plasma hormone levels could affect the relative rates of distribution and metabolism of the two hormones in the rabbit was examined in experiments in which a tracer (0·4 or 0·2 μg) or a carrier (50 μg) dose of hormone was administered and the urinary and faecal excretion studied. Blood levels at 2, 24 and 48 hr after injection of a tracer dose were very close to those seen in the previous experiment; after a carrier dose the level of T_4 was approximately the same as in the tracer experiment and still considerably greater than that of T_3, although the level of this compound was above that seen when a tracer dose was given (Table 6). This is consistent with the half-lives in the body determined from the cumulative urinary and faecal excretion. The half-lives of tracer and carrier T_4 were very similar (27 and 29 hr respectively) but the T_3 experiments gave a value of 26 hr for the tracer dose and a rather longer half-life (34 hr) for carrier T_3. Again there appears to be no change in the

initial distribution of the hormones, as judged by the 2 hr level in the blood, or in the biological half-life in the body that can be related to the pattern of protein binding or that can explain the similarity in biological activity.

An alternative explanation may be available from a consideration of the pathways by which the rabbit disposes of an excess of thyroid hormone. It appears to differ in this from the rat. In the rat a large dose of thyroxine is eliminated to a greater extent in the faeces than is a small, physiological one, owing to an increased rate of biliary secretion as the plasma level of thyroxine is raised (Myant, 1957). In contrast, the biliary clearance of T_3 in the rat is already maximal at normal plasma levels and is unchanged as this level is increased. An animal such as the rat, in which biliary clearance and faecal excretion is the main means of dealing with an excess of hormone, should be better able to handle an excess of T_4 than of T_3. This is in fact found to be the case; Maclagan & Wilkinson (1954) showed that, whereas the percentage urinary excretion of ^{131}I (as iodide) after the administration of increasing doses of T_3 and T_4 in the rat remained constant, the percentage excreted in the faeces at 24, 48 and 120 hr after injection increased markedly as larger doses of T_4 were given, but remained constant after giving T_3, despite similar increases in the dose administered. Thus the biological half-life of T_4 decreases with increasing doses, but not that of T_3.

An apparently greater biological potency for T_3 could well arise from these differences. The rabbit does not appear to behave at all like the rat in dealing with an excess of thyroid hormone. In confirmation of the previous findings of Brown-Grant & Gibson (1955), when a large dose of labelled T_4 is administered to rabbits, it is the proportion of the ^{131}I excreted in the urine that is increased by comparison with the pattern of excretion seen after giving a tracer dose (Fig. 6). Similarly, an increase in urinary and a decrease in faecal excretion is seen when the metabolism of a large dose of T_3 is compared with that of a tracer dose (Fig. 5). Again, in contrast to the findings in the rat, the biological half-life of T_4 is not decreased when the dose is increased and in the case of T_3 the half-life after a large dose may even be slightly lengthened.

The explanation suggested for the finding that T_3 and T_4 are equipotent in birds, namely that there is no specific inter-α-globulin TBG and that consequently both hormones reach the tissues at equal rates, is not applicable to the similar equipotency observed in the rabbit. At least when the two hormones are compared in an assay depending on the maintenance of an excess of hormone in the body, their equipotency appears to result from the manner in which they are metabolized. Whereas in the rat the process of biliary secretion appears to be the limiting factor in the metabolism of the hormones, in the rabbit deiodination by the tissues seems to

be the most important means of dealing with an excess of hormone. The tissues of the rabbit contain a thyroxine dehalogenase which is capable of dealing with far larger amounts of both T_3 and T_4 than are normally available as substrate (Tata, 1960*b*). The equipotency of T_3 and T_4 may be related to the existence of a large reserve capacity for dealing with an excess of either hormone in the rabbit in contrast to the limited capacity of the major excretory pathway for T_3 in the rat.

The over-all effectiveness of the biliary-faecal excretory pathway for thyroid hormones in the rabbit is low. Whether this is the result of a low rate of biliary secretion or the high efficiency of reabsorption, either on the first passage through the gut or as a result of refection is not known. The relatively short half-life suggests that the first explanation is more probable.

SUMMARY

1. The distribution of a physiological (2 μg) dose of ^{131}I-labelled T_3 and T_4 in the rabbit has been studied.

2. At 30 min after injection the volume of distribution was 837 ml. in the case of T_3 and 110 ml. in the case of T_4 as compared with an estimated plasma volume of 83 ml.

3. When the hormone content of a wide variety of tissues was determined 30 min after injection it was found that the T_3 content was always higher than that of T_4.

4. No evidence for a specific concentration of T_3 in any organ was obtained; the pattern of distribution followed that of T_4. The liver and muscle were quantitatively the most important sites.

5. The distribution of normal and increased amounts of T_3 and T_4 was studied by paper electrophoresis of serum in barbiturate buffer. Rabbit serum, like that of other mammals, contains a specific α-globulin thyroxine-binding globulin with a higher affinity for T_4 than for T_3. The capacity of this protein is less than that of the TBG of human plasma.

6. The half-lives of T_3 and T_4 in the plasma between 25 and 96 hr after injection were estimated to be 27 hr. The half-life of a similar physiological dose was determined by measuring the rate of loss from the body in the urine and faeces and was found to be 26 hr for T_3 and 27 hr for T_4. When an excess of hormone (50 μg) was injected, the half-lives determined in this way were 34 and 29 hr respectively.

7. A difference in the pattern of excretion after a large dose of either hormone was noted. Urinary excretion was increased, suggesting that a greater degree of deiodination was occurring after the injection of an excess of hormone.

8. The pattern of binding to the serum proteins does not explain the

anomalous equipotency of T_3 and T_4 in the rabbit. This may be related to the observation that an excess of hormone is dealt with by deiodination.

Our thanks are due to Mr Melvin Sherwood for his valuable assistance in carrying out these experiments.

REFERENCES

Armin, J., Grant, R. T., Pels, H. & Reeve, E. B. (1952). The plasma, cell and blood volumes of albino rabbits as estimated by the dye (T1824) and ^{32}P marked cell methods. *J. Physiol.* **116**, 59–73.

Balfour, W. E. & Tunnicliffe, H. E. (1960). Thyroxine binding by serum proteins. *J. Physiol.* **153**, 179–198.

Bartlett, S., Burt, A. W. A., Folley, S. J. & Rowland, S. J. (1954). Relative galactopoietic effects of 3:5:3′-triiodo-L-thyronine and L-thyroxine in lactating cows. *J. Endocrin.* **10**, 193–201.

Block, R. J., Durrum, E. L. & Zweig, G. (1955). *A Manual of Paper Chromatography and Electrophoresis.* New York: Academic Press, Inc.

Brown-Grant, K. (1955). A comparison of thyroxine and triiodothyronine as inhibitors of pituitary thyrotrophic hormone secretion in the rabbit. *J. Physiol.* **127**, 352–357.

Brown-Grant, K. (1960). The hypothalamus and the thyroid gland. *Brit. med. Bull.* **16**, 165–169.

Brown-Grant, K. & Gibson, J. G. (1955). The metabolism of exogenous and endogenous thyroid hormone in the rabbit. *J. Physiol.* **127**, 341–351.

Bruce, H. M. & Parkes, A. S. (1947). Feeding and breeding of laboratory animals. III. Observations on the feeding of guinea-pigs. *J. Hyg., Camb.*, **45**, 70–87.

Gilliland, I. C. & Strudwick, J. I. (1953). Comparison of the effect of equimolecular solutions of L-thyroxine and L-triiodothyronine in blocking TSH secretion in the chick's pituitary. In The Thyroid Gland. *Mem. Soc. Endocrin.* **1**, 14–16.

Gross, J., Ford, D. F., Symchowicz, S. & Horton, J. H. (1957). The distribution and metabolism of thyroid hormones. *Ciba Foundation Colloquia in Endocrinology*, **10**, 182–189.

Ingbar, S. H. (1958). Pre-albumin: a thyroxine-binding protein of human plasma. *Endocrinology*, **63**, 256–259.

Maclagan, N. F. & Wilkinson, J. H. (1954). Some differences in the metabolism of thyroxine and triiodothyronine in the rat. *J. Physiol.* **125**, 405–415.

Myant, N. B. (1957). Relation between the biliary clearance rate of thyroxine and the binding of thyroxine by serum proteins. *J. Physiol.* **135**, 426–441.

Myant, N. B. (1958). The passage of thyroxine and triiodothyronine from mother to foetus in pregnant rabbits, with a note on the concentration of protein-bound iodine in foetal serum. *J. Physiol.* **142**, 329–342.

Myant, N. B. & Osorio, C. (1959). Serum proteins, including thyroxine binding proteins, in maternal and foetal rabbits. *J. Physiol.* **146**, 344–357.

Newcomer, W. S. (1957). Relative potencies of thyroxine and triiodothyronine based on various criteria in thiouracil-treated chickens. *Amer. J. Physiol.* **199**, 413–418.

Pipes, G. W., Premachandra, B. N. & Turner, C. W. (1959). The biological half-life of L-thyroxine and L-triiodothyronine in the blood of dairy cows. *J. Dairy Sci.* **42**, 1606–1614.

Pitt-Rivers, R. & Tata, J. R. (1959). *The Thyroid Hormones.* London: Pergamon Press.

Robbins, J. & Rall, J. E. (1957). The interaction of thyroid hormones and protein in biological fluids. *Recent Progr. Hormone Res.* **13**, 161–202.

Shellabarger, C. J. (1955). A comparison of triiodothyronine and thyroxine in the chick goiter-prevention test. *Poultry Sci.* **34**, 1437–1440.

Tata, J. R. (1960*a*). Transport of thyroid hormones. *Brit. med. Bull.* **16**, 142–147.

Tata, J. R. (1960*b*). The partial purification and properties of thyroxine dehalogenase. *Biochem. J.* **77**, 214–226.

Tata, J. R. & Shellabarger, C. J. (1959). An explanation for the difference between the responses of mammals and birds to thyroxine and triiodothyronine. *Biochem. J.* **72**, 608–613.

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THE LOCAL METABOLIC ACTION OF ADRENALINE ON SKELETAL MUSCLE IN MAN

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The relation between the vascular and the metabolic effects of adrenaline has recently been emphasized by Lundholm (1956), who has advanced the proposition that the dilator action of adrenaline on the vessels of skeletal muscle is indirect and mediated by release of lactic acid. Although it is well known that adrenaline promotes lactic acid formation in animal muscle (Griffith, 1951; Lundholm, 1956), evidence on this point in the human is lacking. Several investigators have suggested that adrenaline may release lactic acid from human skeletal muscle (Barcroft & Cobbold, 1956) but Hildes, Purser & Sherlock (1949) were unable to demonstrate a direct metabolic action of adrenaline on the muscles of the lower limb in man. They could find no increase in the lactic acid level of the femoral venous blood and no reduction in glycogen content of biopsy specimens from gastrocnemius muscle following infusions of adrenaline into the femoral artery.

Using adrenergic blocking agents, de la Lande & Whelan (1959) have demonstrated a potent dilator action of adrenaline when administered locally in the forearm, and the findings are consistent with the view that a metabolite released from muscle might be responsible for this effect.

The possibility of a direct local metabolic effect of adrenaline on human muscle has been re-examined in the upper limb where more precise correspondence of infused tissue and sampling site can be ensured than in the leg. Changes in the levels of a number of metabolites have been found in the venous effluent, which indicate a direct glycogenolytic action of adrenaline on human skeletal muscle.

METHODS

The subjects were ourselves, our colleagues and volunteer medical students. The laboratory was maintained at a temperature of 23–24° C and the subject rested on a couch for at least 30 min before observations began.

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A polythene catheter ('Intracath', Bard) was inserted through a wide-bore needle into an antecubital vein of one arm and passed down into a deep vein carrying blood from forearm muscle (Roddie, Shepherd & Whelan, 1956) and maintained free from clotting by the occasional injection of 0·1 ml. heparin in NaCl 0·9 g/100 ml. (25 i.u./ml.). In a few experiments a catheter was also inserted into a superficial vein draining blood from the skin of the forearm. The oxygen saturation of samples of blood withdrawn through the catheter before, during and after a period of rhythmic exercise lasting one minute served to check that the catheter was inserted into a vein draining muscle. The oxygen saturation of the samples was measured by the spectrophotometric method described by Roddie, Shepherd & Whelan (1957). The deep-muscle blood saturation showed a sharp fall during exercise and rose above the resting value after exercise ceased.

A 23 gauge, short-bevel needle was next inserted into the brachial artery at the elbow through an area of skin anaesthetized by 2% lignocaine, and was connected by a length of polythene tubing to a constant-infusion apparatus which delivered NaCl 0·9 g/100 ml., containing ascorbic acid (1:50,000), at a rate of 1 ml./min.

A water-filled venous occlusion plethysmograph at 35° C was then applied to the forearm and blood-flow recordings begun, the circulation through the hand being arrested by a pneumatic cuff at the wrist inflated to 200 mm Hg.

After a control period of 10–15 min, during which 3 or 4 flow measurements were made each minute, an infusion of adrenaline in ascorbic-acid saline, in a dose of 0·05 μg/min was given into the brachial artery for a period of 10 min, flow recordings being continued throughout. This infusion demonstrated that the forearm was behaving in a characteristic fashion to adrenaline (Whelan, 1952).

Thirty minutes was allowed to elapse for recovery from this adrenaline infusion. The plethysmograph remained in position throughout the experiments, to maintain the temperature of the forearm segment. Samples of muscle venous blood were taken through the catheter into nylon syringes, for estimation of the levels of blood lactic acid, blood pyruvic acid, blood phosphate and plasma potassium. Two or three such groups of resting samples were taken with an interval of 5 min, and then an infusion of adrenaline (0·05 μg/min) was given for 10 min and sampling continued at various intervals during the infusion and in the 10 min after it was discontinued. Flow measurements were not made during this infusion, as it was desired to avoid venous congestion in the sampling periods, but the wrist cuff was inflated throughout to 200 mm Hg to exclude the hand circulation.

After a further interval of 10–15 min a third infusion of adrenaline made up in 0·9% NaCl containing Evans Blue dye (T 1824) (0·5 mg/min), or an infusion of dye alone, was given into the brachial artery and samples of the muscle blood withdrawn through the catheter before and during the infusion. These samples were centrifuged and the plasma examined for dye content.

In a number of experiments the infusion of adrenaline during which blood flow was measured, and the forearm exercise test for position of the catheter, were both carried out at the end of the experiment, and the venous sampling during adrenaline infusion was made before any muscle activity or previous adrenaline infusion. Similar results were obtained in both patterns of experiment. In a few of the experiments samples of brachial artery blood were taken in the preliminary resting period and immediately after the infusion of adrenaline ceased. Arterial samples were not taken during the infusion period, as this would have necessitated interruption of the infusion.

Blood lactic acid was measured by the method of Barker & Summerson (1941), phosphate by the method of Fiske & SubbaRow (1925), pyruvic acid by the method of Friedemann & Haugen (1943) and plasma potassium by flame photometry. For the phosphate estimations blood samples were weighed for greater accuracy and values were reproducible to within 0·1 mg/100 ml.

RESULTS

Figure 1 shows the concentrations of lactic acid in the venous effluent from the forearm muscles before, during and after infusions of adrenaline into the brachial artery in six different subjects. In every case there was a gradual rise during the infusion to a peak level which ranged from 36 to 173% above the resting level. In two cases the highest value was not obtained until some minutes after the infusion ceased. The concentration of lactic acid returned only slowly to the resting level and was still elevated 10–20 min after the end of the infusion.

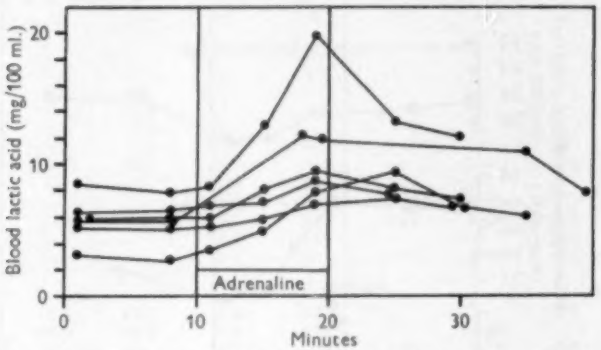

Fig. 1. Concentrations of lactic acid in the venous blood returning from the forearm muscles in six different subjects before, during and after infusion of adrenaline into the brachial artery. Adrenaline (0·05 μg/min) was infused between the two vertical lines.

The changes observed in the concentrations of the other metabolites in venous blood during adrenaline infusion were as follows: the concentration of pyruvic acid rose (3 experiments) while that of phosphate fell (5 experiments). Potassium showed a transient rise, corresponding in time to the period of transient vasodilatation, and then fell below the initial resting level for the remainder of the infusion (7 experiments). The results of a typical experiment illustrating the above changes, together with changes in forearm blood flow, are shown in Fig. 2. The 'after-dilatation' (Whelan, 1952) was seen following most infusions and blood flow only returned to the pre-infusion resting level after 10–20 min during which the metabolites also returned towards resting levels.

In Table 1 all the data on all the subjects are shown. The resting value (*A*) is the mean of the control values taken during the 10 min period before the adrenaline infusion began. The value (*B*) is that obtained when the change from resting was at its greatest, which in most cases was during

the last 3 min of the adrenaline infusion, but in a few was in the first 5 min after the infusion ceased. In every case the pattern of change in the metabolites measured was the same as that in TR, illustrated in Fig. 1, differences being quantitative only. The arterial concentrations were not

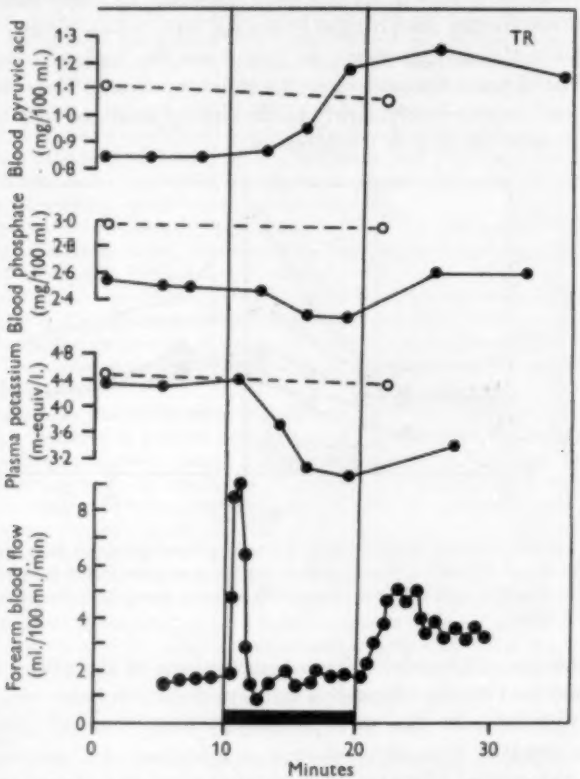

Fig. 2. The changes in the venous effluent from the forearm muscles in blood pyruvic acid, blood phosphate, plasma potassium and forearm blood flow during infusion of adrenaline (0·05 μg/min) into the brachial artery in subject TR. O - - - O = values for brachial arterial blood for each of the constituents mentioned. The infusion of adrenaline is indicated by the black rectangle.

measured in every experiment but sufficient determinations were made to indicate that a slight positive arteriovenous difference existed in pyruvic acid, phosphate and potassium in the initial 'resting' period. At the height of the changes caused by adrenaline the venous levels of lactic acid and pyruvic acid rose above the corresponding arterial levels, indicating an increased output of these constituents from the muscles. In the case of

potassium and phosphate a positive uptake occurred, since the arterio-venous differences increased during the adrenaline infusion.

In one experiment (ISD(3)) in which blood samples from a superficial vein draining the skin of the forearm were taken at the same time as samples from a deep-muscle vein during intra-arterial adrenaline, no changes occurred in the lactic, pyruvic or potassium levels of the superficial

TABLE 1. Effect of intra-arterial infusions of adrenaline (0·05 μg/min) on venous blood levels of metabolic products from forearm muscle and skin

	Lactic acid (mg/100 ml.)		Potassium (m-equiv/l.)		Pyruvic acid (mg/100 ml.)		Phosphate (mg/100 ml.)	
Subject	*A*	*B*	*A*	*B*	*A*	*B*	*A*	*B*
				Muscle blood samples				
AH	6·0	9·7	—	—	—	—	—	—
ISD (1)	2·9	8·0	—	—	—	—	—	—
JG	8·2	19·9	—	—	—	—	—	—
MD	5·3	7·2	—	—	—	—	—	—
AGS	6·3	9·2	3·8	3·1	—	—	—	—
JM	5·6	12·0	3·8	2·7	—	—	2·5	1·3
ISD (2)	—	—	3·9	3·4	—	—	—	—
RS	—	—	3·9	3·4	0·42	0·92	3·1	3·0
TR	—	—	4·4	3·0	0·85	1·26	2·5	2·3
BP	—	—	3·6	3·0	—	—	2·6	2·4
ISD (3)	—	—	4·9	3·7	0·71	1·04	3·3	3·1
				Skin blood samples				
ISD (3)	10·5	11·1	4·4	4·5	—	—	3·3	3·3
PH	13·6	13·9	4·1	4·0	1·70	1·35	—	—
LRM	—	—	4·1	4·2	1·32	1·22	2·5	2·5

A, mean of 2 samples during pre-infusion period; *B*, value obtained during the last 3 min of adrenaline infusion or within a few minutes thereafter, when the change from *A* was at its maximum.

blood while the usual changes in these metabolites were observed in the muscle blood. In two experiments superficial blood samples only were taken, and no changes in metabolite levels were produced by adrenaline infusions. In each experiment Evans Blue dye was detected in the returning venous blood on subsequent infusion of adrenaline solution containing the dye.

DISCUSSION

The changes in the levels of lactic acid, potassium, pyruvic acid and phosphate in the venous effluent from the forearm muscles during intra-arterial infusions of adrenaline are indicative of a direct effect of adrenaline on the metabolism of human skeletal muscle. The finding is directly contrary to the observations of Hildes *et al.* (1949), who could detect no changes in metabolite concentrations in the femoral venous blood during infusions of adrenaline into the femoral artery in the groin nor any reduction in glycogen content of biopsy tissue from gastrocnemius muscle.

The indication that adrenaline was in fact perfusing the limb was the blanching of the skin noted during the infusion. There was no evidence, however, to suggest that adrenaline was perfusing the calf muscles, or, if it were, that the femoral vein samples were in fact coming from the same area of the calf, nor that the biopsies were taken from an area perfused by adrenaline or drained by the femoral vein.

All these factors are particularly important in the case of the lower limb, where the site of injection of adrenaline and of sampling lay at a considerable distance from the muscle concerned. The opportunities for localization of an arterially infused solution into an area of the thigh, for example, are considerable, as is evidenced by flushing confined to distinct areas usually on the lateral or medial aspect of the thigh during femoral intra-arterial infusions of vasodilator drugs (Marshall & Whelan, 1956).

In the present series of experiments the upper limb was used and the site of arterial infusion was much closer to the muscle group under study. Localization of the infused drug in a small area, however, can still occur in the upper limb. That it did not do so in the experiments described is demonstrated, first, by the fact that the characteristic blood-flow changes always occurred in the forearm segment immediately distal to the needle and, secondly, by the fact that dye injected along with adrenaline into the artery was recovered from the blood sampled by the catheter. That a catheter inserted as described into a deep forearm vein samples mainly muscle venous blood has been shown by Roddie *et al.* (1956), and was checked in each experiment by the changes in venous oxygen saturation accompanying exercise of the forearm muscles.

No correction of the values obtained for lactic acid and other substances assayed was made for changes in blood flow accompanying the adrenaline infusions, since with the dose chosen (0·05 μg/min) the blood flow through the forearm segment during the greater part of the infusion was little different from the resting value. Griffith, Lockwood & Loomis (1946) were able to demonstrate increases in venous blood lactic acid on intra-arterial infusion of adrenaline in the perfused limb of the cat only if the dose did not produce marked vasoconstriction in the limb. Adrenaline given by this route into the human arm is known to produce vasoconstriction of the vessels of the hand, which is mainly skin (Barcroft & Swan, 1953). Evidence about the effect of intra-arterial adrenaline on skin and muscle vessels in the human forearm is lacking. If the forearm skin vessels were constricted in the present experiments, the fact that the total forearm blood flow showed little or no change may indicate that the muscle blood flow was increased. Such an increase would tend to dilute the concentration of any metabolite released from the muscles and thus lead to an underestimate of the amount released, but it would also have the effect of causing

an apparent rise in the concentration of any substance with a positive 'resting' arteriovenous difference by tending to move its venous concentration towards the arterial level. It could not, however, account for a rise above the arterial level, such as occurred with lactic and pyruvic acids, nor for a fall in venous concentration, as occurred in the case of potassium and inorganic phosphate. The finding that no changes occurred in the metabolite levels of blood returning from a superficial vein draining skin, at a time when there were changes in the muscle blood levels, indicates that the metabolic action of adrenaline is confined to the muscle and also that the changes, for example, in plasma potassium could not be accounted for by any action of adrenaline in causing shifts of this electrolyte between the plasma and the red cells.

In the case of lactic and pyruvic acids the findings are in accord with other evidence that adrenaline promotes an increase in glycogenolytic activity in muscle (Ellis, 1956, 1959). The changes in potassium and phosphate may also be linked with glycogenolysis, but direct biochemical evidence on this point is lacking. An ability of adrenaline to depress potassium and phosphate loss from skeletal muscle *in vitro* and to promote potassium influx in smooth muscle and uptake of phosphate into muscle *in vivo* has been reported by a number of investigators (Ellis, 1959). Hence the metabolic effects of adrenaline which have been observed in the human in the present experiments are consistent with the observations on other species *in vivo* and on isolated tissues *in vitro*.

The present finding that the 'after-dilatation' seen in the forearm following cessation of intra-arterial infusion of adrenaline subsides over a period of 10–20 min, and is paralleled by the return of the metabolite levels towards their respective base-line values suggests that the two phenomena are intimately related. Lundholm (1956) has proposed on the basis of animal studies that the glycogenolytic action of adrenaline may be related to its dilator effect on vascular smooth muscle, but whether lactic acid, or indeed any of the products of the metabolic action of adrenaline, participates in the vascular responses in the human forearm remains to be decided.

SUMMARY

Infusions of adrenaline into the brachial artery in man cause an increase in glycogenolytic activity in the forearm muscles which is manifested by an increase in lactic and pyruvic acid and a fall in potassium and phosphate levels in the effluent venous blood.

We are indebted to our colleagues and students who volunteered as subjects for this study, and to Mr A. McNeil for technical assistance and for carrying out most of the blood lactic-acid determinations.

REFERENCES

Barcroft, H. & Cobbold, A. F. (1956). The action of adrenaline on muscle blood flow and blood lactate in man. *J. Physiol.* **132**, 372–378.

Barcroft, H. & Swan, H. J. C. (1953). *Sympathetic Control of Human Blood Vessels.* Physiological Society Monographs. London: Arnold and Co.

Barker, S. B. & Summerson, W. H. (1941). The colorimetric determination of lactic acid in biological material. *J. biol. Chem.* **138**, 535–554.

De la Lande, I. S. & Whelan, R. F. (1959). The effect of antagonists on the response of the forearm vessels to adrenaline. *J. Physiol.* **148**, 548–553.

Ellis, S. (1956). The metabolic effects of epinephrine and related amines. *Pharmacol. Rev.* **8**, 485–562.

Ellis, S. (1959). Relation of biochemical effects of epinephrine to its muscular effects. *Pharmacol. Rev.* **11**, 469–479.

Fiske, C. H. & SubbaRow, Y. (1925). The colorimetric determination of phosphorus. *J. biol. Chem.* **66**, 375–400.

Friedemann, T. E. & Haugen, G. E. (1943). The determination of keto acids in blood and urine. *J. biol. Chem.* **147**, 415–442.

Griffith, F. R. Jr. (1951). Fact and theory regarding the calorigenic action of adrenaline. *Physiol. Rev.* **31**, 151–187.

Griffith, F. R., Jr., Lockwood, J. E. & Loomis, T. A. (1946). The effect of intra-arterially injected adrenaline on blood flow, sugar retention and lactate output of the leg tissues of anaesthetised cats. *Amer. J. Physiol.* **146**, 677–688.

Hildes, J. A., Purser, S. H. & Sherlock, S. (1949). The effects of intra-arterial adrenaline on carbohydrate metabolism in man. *J. Physiol.* **109**, 232–239.

Lundholm, L. (1956). The mechanism of the vasodilator effect of adrenaline. 1. The effect on skeletal muscle vessels. *Acta physiol. scand.* **39**, Suppl. 133.

Marshall, R. J. & Whelan, R. F. (1956). Intra-arterial oxygen in peripheral vascular disease. *Brit. med. J.* ii, 1448–1451.

Roddie, I. C., Shepherd, J. T. & Whelan, R. F. (1956). Evidence from venous oxygen saturation measurements that the increase in forearm blood flow during body heating is confined to the skin. *J. Physiol.* **134**, 444–450.

Roddie, I. C., Shepherd, J. T. & Whelan, R. F. (1957). A spectrophotometric method for the rapid estimation of blood oxygen saturation, content and capacity. *J. clin. Path.* **10**, 115–119.

Whelan, R. F. (1952). Vasodilatation in human skeletal muscle during adrenaline infusions. *J. Physiol.* **118**, 575–587.

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STIMULI INFLUENCING THE SECRETION OF ACID BY THE ABOMASUM OF SHEEP

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The acidity of the food material flowing from the abomasum to the duodenum of sheep varies within comparatively narrow limits, and under the conditions of stall-feeding, when the animals are fed on dry fodder and meals, the pH values are usually between 2 and 3. In contrast, the amount of acid secreted by innervated pouches of the fundic region of the abomasum may fluctuate widely. These observations suggest that the amount of acid secreted is the resultant of excitatory and inhibitory factors which operate to produce a comparatively stable reaction of the food material leaving the abomasum. On a broad basis, the factors likely to be concerned are the amount and composition of the predigested food flowing into the abomasum from the omasum; another is the rate at which the abomasal contents are discharged into the duodenum.

The manner in which food material passes through the omasum from the reticulo-rumen is still obscure but there is reason to believe that fluid flows through the omasum more rapidly than the solid matter, so that the organ functions as a sieve. Knowledge of the composition of the food material flowing into the abomasum is meagre. Oyaert (1955) found that the predigested food flowing from the omasum of sheep contained 5–10% of finely divided dry matter and 20–30 m-mole CO_2/l. and the pH was 6·8–7·3.

During extremes of fasting and feeding and on a variety of diets, the concentration of steam-volatile fatty acid *in the food material leaving the abomasum* was 4–30 mM (Masson & Phillipson, 1952; Ash, 1961). These concentrations are low compared with those in rumen fluid but it should be realized that approximately half the material leaving the abomasum is abomasal secretion which contains little or no fatty acid. Furthermore, fatty acid may be absorbed as the food material flows through the abomasum. The concentration of fatty acid *in the food material flowing into the abomasum* will depend on the concentration in the reticulo-rumen and the amount absorbed as the fluid flows through the omasum. Recent work at this Institute has shown that the predigested food material flowing into the abomasum from the omasum of sheep contains 30–60 mM fatty acid (P. K. Briggs, personal communication).

This paper describes experiments which were designed to investigate the effect on acid secretion of some of the factors considered above, special attention being given to the influence of fatty acid in the abomasum. A preliminary account of some of the experiments has been given to the Physiological Society (Ash, 1959*b*).

METHODS

Animals. The experiments were carried out on six sheep with innervated abomasal pouches. All the sheep were fitted with rumen cannulae and three had pyloroduodenal re-entrant fistulae. A detailed description of the surgical preparation and maintenance of the animals has been given (Ash, 1961). A small Perspex cannula was inserted approximately 10 cm from the pylorus and into the duodenum of another sheep with a pouch.

Preparation of the animals for experiment. In order to prevent predigested food from entering the abomasum during the experiments the reticulo-rumen was emptied. The sheep were deprived of food for 24–48 hr, during which time they were allowed free access to water. On the morning of, or less frequently the night before, an experiment a Perspex tube, bore 1·0 cm, was inserted through the rumen cannula and the contents of the organ were removed by suction. The tube was connected to a 5 l. aspirator which was evacuated from a water pump. This method avoided manipulation of the abdominal wall (Ash, 1959*a*), but care was required to prevent sucking the rumen wall against the end of the tube. After the majority of the material had been removed the interior of the organ was rinsed with 5–10 l. of isotonic NaCl solution or tap water at 39° C. Residual fluid was removed from the reticulum and ventral sac of the rumen as thoroughly as possible without disturbing the animal unduly. When the rumen was emptied the night before an experiment, 4 l. of isotonic NaCl solution, or a phosphate buffer containing 100 m-mole fatty acid/l. (pH 6–7), was returned to the rumen. On the morning of the experiment the solution was drained from the rumen by suction.

The contents of the abomasum were allowed to drain away through the cannula in the main body of the organ and the interior was rinsed with isotonic NaCl solution. In those sheep fitted with pyloroduodenal re-entrant fistulae the cannulae were disconnected and the duodenal cannulae plugged.

Although the reticulo-rumen and the abomasum were emptied it must be assumed that the omasum contained some food material in all the experiments. The contents of the omasum consist of vegetable material (approximately 18% dry matter) held between the omasal leaves and it is difficult to know the effect this has on acid secretion by the abomasum. Although the abomasum was rinsed repeatedly before each experiment, a few of the test solutions drained from the organ contained rather more solid material than was to be expected simply from dislodgement of residual material from between the mucosal flaps of the abomasum.

During the experiments the sheep were held in a recording stand. The attachment of tubes to any of the cannulae was made at the beginning of the experiment and manipulations during the course of an experiment were reduced to a minimum. The most complicated arrangement was that shown in Fig. 1.

All the sheep used in the experiments were thoroughly trained to the experimental conditions. The feeding and daily routine associated with the maintenance of the animals was carried out by people directly concerned with the experiments and only on rare occasions were the sheep ever handled by others and then it was usually in the presence of an experimenter.

At the end of an experiment, rumen contents were returned to the reticulo-rumen and 500 ml. of isotonic NaCl solution was introduced into the abomasum. The pyloroduodenal

re-entrant cannulae were reconnected and all other cannulae plugged. Not less than 8 days elapsed between experiments on the same sheep and usually it was longer. Except for a temporary increase in water consumption on the day following some of the experiments there appeared to be no observable effects.

Analytical. The methods used for estimating titratable acid and steam-volatile fatty acid have been described (Ash, 1961). All the acid concentrations in the secretions and outputs were calculated from phenolphthalein titrations. In some experiments Töpfers reagent or

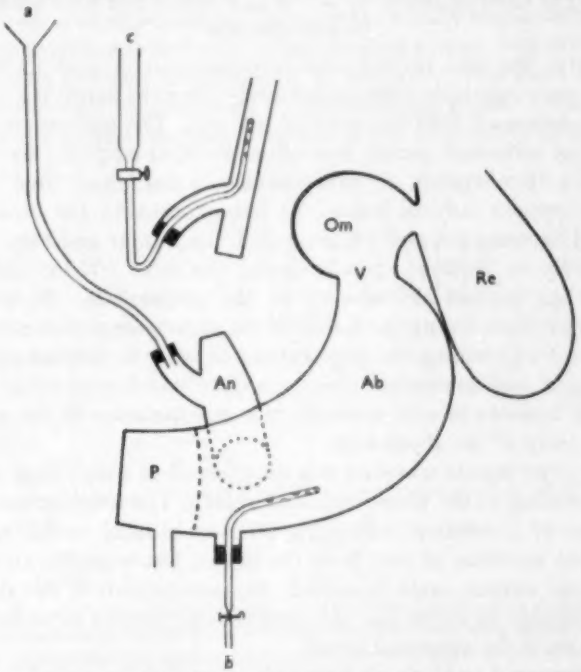

Fig. 1. Diagram of the 'isolated' abomasum preparation. Test solutions were introduced into the organ through the funnel *a*, and samples were obtained from the tube *b*; *c*, burette for infusing solutions into the duodenum. Re, reticulum; Om, omasum; Ab, main body of abomasum; An, pyloric part of abomasum; P, pouch. The omaso-abomasal orifice (V) is guarded by mucosal flaps but radiographical studies, although 'not definite enough to warrant a statement' suggest that material may flow from the abomasum to the omasum (Benzie & Phillipson, 1957).

bromthymol blue was used to detect the presence of 'free' acid in the secretions. pH measurements were made with a glass electrode immediately after withdrawing samples from the abomasum and no precautions were taken to prevent loss of CO_2.

The sodium salts and the acids used to make up the test solutions were 'Analar' reagents (Hopkin and Williams, Ltd) or 'Laboratory' reagents (British Drug Houses, Ltd). In all the experiments with fatty acid solutions the ratio acetic:propionic:butyric was 70:20:10. The composition of the fatty acid mixture entering the abomasum during normal feeding will be influenced by the type of fermentation taking place in the rumen and whether or

not preferential absorption of one acid over another occurs in the omasum. The ratio selected for these experiments appeared to be a possible one to occur.

Atropine was injected as the sulphate. L-adrenaline was dissolved in HCl and diluted with NaCl 0·9 g/100 ml. to give solutions containing 5 and 10 μg/ml.

RESULTS

The effect of emptying the reticulo-rumen and abomasum on acid secretion

Typically, the rate of flow and concentration of acid in the pouch secretion were relatively high immediately after the emptying procedure, and then decreased over the next 45–120 min. The concentration in the first 15 min collection period was often 50–80 m-equiv/l., decreasing to usually 2·9–16 m-equiv/l. in subsequent samples when 'free' acid was absent or present only in traces. In one experiment the concentration fluctuated between 3·9 and 8·9 m-equiv/l. for 2·75 hr and then increased progressively to 30–36 m-equiv/l. during the next 1·75 hr, although no stimulus was applied deliberately to the preparation. It became increasingly obvious during the course of the experiments that extreme care was required in handling the preparations in order to prevent non-specific excitation of acid secretion. One procedure which sometimes caused a temporary increase in acid secretion was manipulation of the cannula in the main body of the abomasum.

In four experiments secretion was maintained at a high level in spite of repeated rinsing of the abomasum and rumen. The introduction into the abomasum of a solution containing 50 m-mole fatty acid/l. at pH 2·4 reduced the secretion of acid from the pouch, but when the solution was drained acid output again increased. An examination of the sheep after the experiments in which this occurred usually showed some irritation of the skin round the abomasal fistula.

There appeared to be no advantage in emptying the reticulo-rumen the night before an experiment; it was still necessary to drain and rinse the abomasum on the following morning and this seemed to be responsible for the high initial levels of acid secretion. In a later section experiments are described which suggest that the decrease in acid secretion in some experiments may not have been due entirely to withdrawal of stimulation but partly to inhibition from emptying the reticulo-rumen. In all, 36 experiments were performed on six sheep; 28 of the experiments were successful.

The effect on acid secretion of introducing rumen fluid into the abomasum

Rumen fluid was obtained by straining through gauze the contents removed from the reticulo-rumen after the sheep had been deprived of

food. The fluid was warmed to 39° C and allowed to flow into the abomasum from a reservoir connected to the abomasal cannula. In order to mimic differences in the flow of predigested food material into the abomasum from the forestomach the effect of increasing the volume of the infusion was also investigated. In seven trials on three sheep 250–500 ml. of fluid was infused over 15–35 min; an increase in acid secretion by the pouch was observed in all experiments. The response usually began within 15 min of starting the infusion and acid output reached a peak in 45–90 min; acid secretion then declined progressively or, less often, with short periods of increased secretion superimposed on the declining phase. Increasing the volume of the infusion increased the size of the response (Fig. 2).

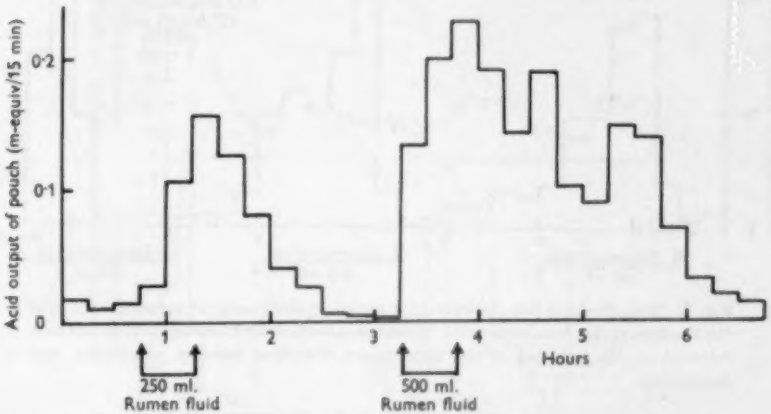

Fig. 2. The effect on acid secretion by a pouch of introducing rumen fluid directly into the abomasum. The fluid was obtained from the sheep after fasting and the arrows indicate the time during which it was infused into the empty abomasum. Conscious sheep, reticulo-rumen empty and free flow from the abomasum to the duodenum. Maximum acid outputs of this pouch during normal feeding were equivalent to 0·20 and 0·49 m-equiv/15 min on diets of 750 g and 1250 g food/day.

Fluid in the abomasum was free to flow into the duodenum and the decline in acid secretion was associated partly with an increase in the acidity of the fluid remaining in the abomasum and partly with a decrease in the volume. In three experiments the pH of the fluid drained from the main body of the organ, when acid secretion by the pouch had declined to a low level, was 1·5–1·8; the residual volumes were 100–250 ml. The initial pH of the rumen fluid was 6·2–6·5; no precautions were taken to prevent loss of CO_2 and the concentration of steam-volatile fatty acid was not determined.

It was essential, however, to eliminate flow into the duodenum as a

possible source of stimulation and to analyse the stimulus into mechanical and chemical components.

Distension of the abomasum as an adequate stimulus of acid secretion

A condom attached to a length of polythene tubing was introduced into the main body of the abomasum through the cannula and the mouth of the cannula was plugged. Measured volumes of NaCl 0·9°/100 ml. at 39° C were allowed to flow rapidly into the condom from a reservoir.

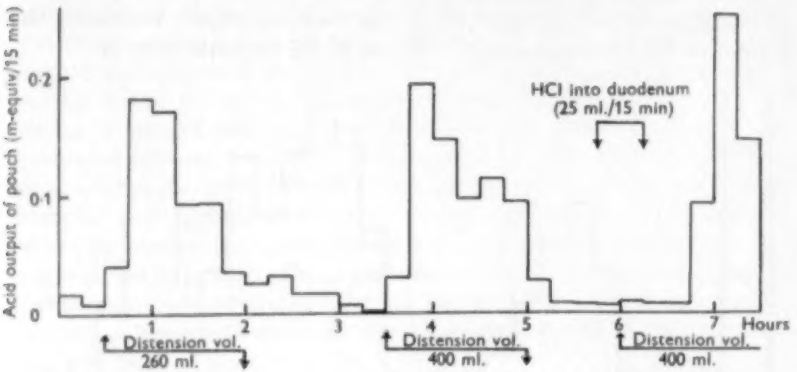

Fig. 3. The effect on the response of a pouch to distension of infusing acid into the duodenum (for details see text). Conscious sheep, reticulo-rumen and abomasum emptied on the morning of the experiment; free flow between abomasum and duodenum.

The introduction of 250–800 ml. of saline into the condom stimulated acid secretion by the pouch. In twelve out of sixteen trials on four sheep the response began within 15 min, reached a peak after 30–45 min and then declined to a low level, although the stimulus was applied for 90 min; large volumes did not appear to increase or prolong the response consistently. When the saline was drained from the condom any residual secretion by the pouch rapidly decreased and variable amounts of fluid flowed from the main body of the abomasum when the plug was removed from the abomasal cannula at the end of a period of stimulation. The titratable acid of the fluid ranged from 18 to 62 m-equiv/l. and on occasions contained bile pigment.

The decline in the response which occurred after 30–45 min, irrespective of the distension volume, was probably caused by inhibition from an accumulation of acid in the abomasum or duodenum rather than by adaptation to distension. This was investigated in two ways. First, an isotonic solution of NaCl-HCl (total acid 62–67 m-equiv/l.) was dripped

into the duodenum through a cannula situated 10 cm from the pylorus. The infusion (25 ml./15 min) began 15 min before and continued for the first 15 min of a period of distension. This procedure delayed the onset of the response but did not prevent a typical response occurring subsequently

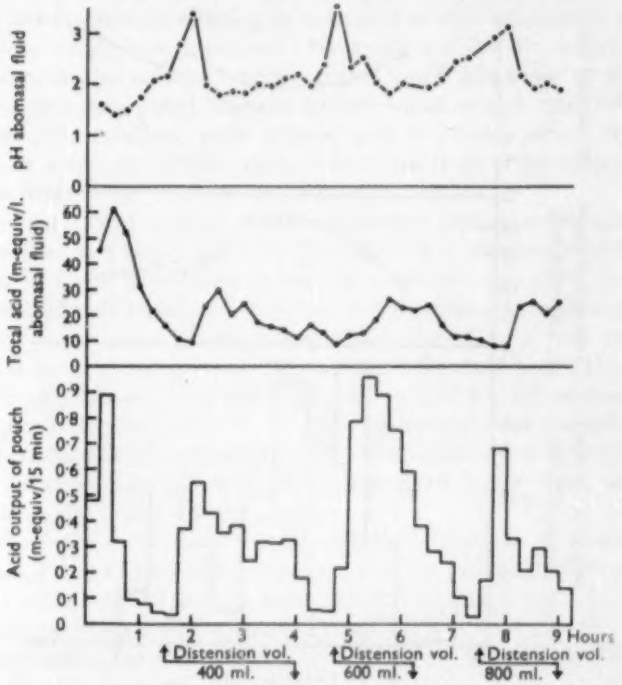

Fig. 4. The effect on acid secretion by a pouch of distending the abomasum with a saline-filled balloon. Top and centre panels show the pH and total titratable acidity respectively of the free fluid in the main body of the abomasum. Acid secretion by the pouch was not increased consistently by increasing the distension volume and appeared to be inhibited on each occasion when the pH value of the abomasal fluid decreased to about 2·0. Conscious sheep, reticulo-rumen and abomasum emptied the night before the experiment; free flow between abomasum and duodenum.

(Fig. 3). Secondly, small samples of fluid were withdrawn from the main body of the abomasum throughout periods of distension. During the initial stages of stimulation the increase in acid output by the pouch was accompanied by an increase in titratable acid and a decrease in pH of the free fluid in the abomasum. As the acidity of the fluid increased the secretion of acid by the pouch began to decline (Fig. 4). The acidity at

which inhibition occurred ranged from 81 m-equiv/l. (pH 1·2) to 23·4 m-equiv/l. (pH 2·1).

In three experiments distension was maintained for 2–3·75 hr, during which time a secondary increase in acid secretion by the pouch was

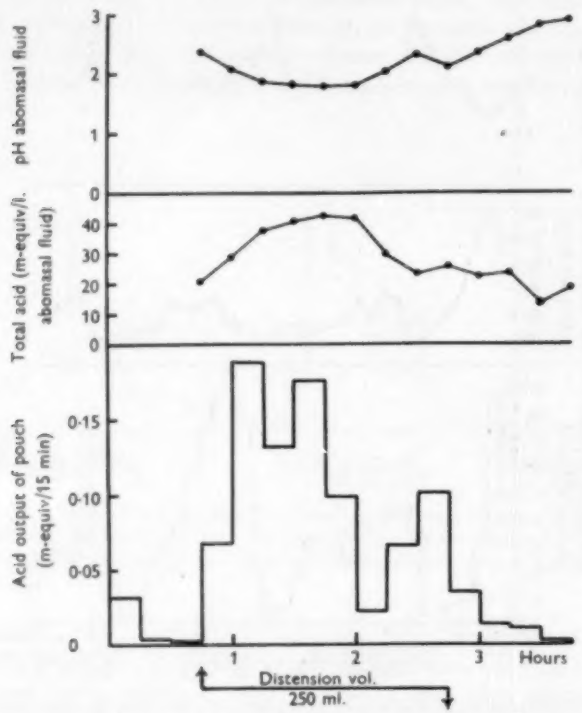

Fig. 5. A secondary increase in acid secretion by a pouch during a maintained period of distension. Top and centre panels show the pH and titratable acidity respectively of the free fluid in the main body of the abomasum. A typical response by the pouch (bottom panel) occurred during the first 90 min of distension and this was followed by a further increase in acid secretion associated with a decrease in acidity of the abomasal fluid. Conscious sheep, reticulo-rumen and abomasum emptied the night before the experiment; free flow between abomasum and duodenum.

observed, and this was associated with a spontaneous decrease in the acidity of the abomasal fluid. When the saline was drained from the condom the secretion of acid by the pouch declined, indicating that distension was still the cause of the secondary rise (Fig. 5 and see also Fig. 4). These experiments indicate that mechanical stimulation of the

main body of the abomasum excites a secretion of acid but for secretion to be maintained acid must not accumulate in the abomasum or duodenum.

The effect of introducing buffered solutions into the abomasum

The effects on acid secretion were compared when approximately isotonic buffered solutions differing in composition were introduced into the abomasum. In any one experiment the volume of the test solutions was kept constant and solutions which caused small responses or failed to stimulate were interposed between stimuli which caused relatively large responses. The solutions were infused over a 15 min period at 39° C, 5–10 ml. of fluid were withdrawn from the abomasum at intervals and the pH measured.

In the first experiment all the solutions were introduced at pH 6 and a comparison was made between the effect of a phosphate buffer and phosphate buffers containing 25 and 50 m-mole fatty acid/l. All three solutions stimulated acid secretion but the responses were greater when fatty acid was present. Furthermore, the effect of fatty acid appeared to depend on its concentration. The acidity of the fluid in the abomasum increased progressively and when the pH reached 2·5–2·8 acid secretion by the pouch declined (Fig. 6). In this experiment, and one other, bile pigment was present in some of the samples obtained from the abomasum. In both trials a high pH was maintained for a longer time and acid secretion by the pouch was prolonged.

The effect of the pH of the solution introduced into the abomasum was investigated in a further series of experiments. A phosphate-HCl solution (pH 2·1) containing 50 m-mole fatty acid/l. failed to cause a significant increase in acid secretion. The effect of this solution was bracketed between phosphate buffers containing 50 m-mole fatty acid/l. at pH 6, both of which stimulated acid secretion.

These experiments appear to indicate that fatty acids potentiate acid secretion, but the differences in phosphate buffer alone and phosphate plus fatty acid may have been due to differences in the rate at which the solutions flowed out of the abomasum. In order to avoid this criticism the experiments were repeated on sheep in which outflow from the abomasum was prevented by closing the pyloroduodenal re-entrant fistulae. The word potentiation is used to describe the effect of fatty acid, since the introduction of a solution into the abomasum involves mechanical stimulation by distension.

Experiments on the 'isolated' abomasum

In the preparation illustrated in Fig. 1 outflow to the duodenum was prevented and secretions from the upper part of the intestine could not

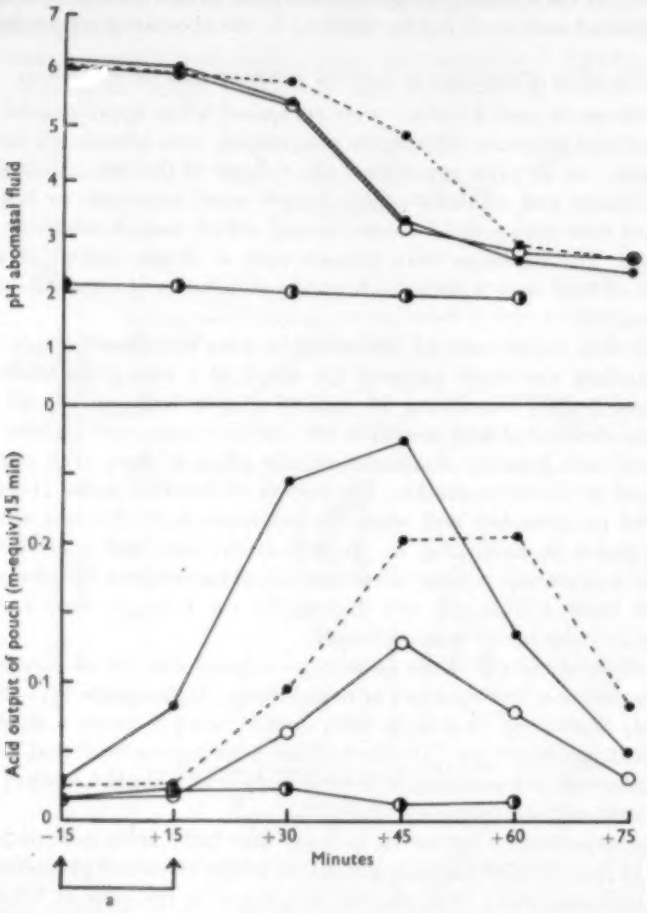

Fig. 6. A comparison of the effects on acid secretion by a pouch (bottom panel) of phosphate buffer alone and phosphate containing fatty acid at two pH values. The solutions, all volumes 300 ml., were introduced directly into the abomasum during the period indicated by the arrows (*a*) and in the following order: ●——● phosphate containing 50 mM fatty acid; ○——○ phosphate buffer alone; ●- - -● phosphate containing 25 mM fatty acid. The effect of phosphate containing 50 mM fatty acid at pH 2·1 ◑——◑ was tested on the same sheep but in a different experiment from the other solutions. The top panel shows the pH of the fluid in the main body of the abomasum; bile pigment was present in the solution marked ●- - -●. Conscious sheep, reticulo-rumen and abomasum emptied on the morning of the experiment; free flow between abomasum and duodenum.

flow back into the abomasum. It was possible that some of the fluid introduced into the abomasum flowed into the omasum. Attempts to establish whether or not this did occur, using phenol red as a marker, were not entirely successful. It is difficult to wash the abomasum of conscious sheep completely free from solid particles, since small amounts cling to the mucosa, especially between the folds. An *in vitro* experiment showed that when solid material which had been centrifuged from normal abomasal contents was washed, dried and added to a solution of phenol red, the concentration of the dye decreased. This indicated that phenol red was bound to solid matter. High recoveries of the dye from solutions introduced into the abomasum were obtained only by repeated rinsing of the organ with large volumes of saline. This was undesirable because of the stimulating effect which the procedure had on acid secretion.

The method of testing the effects of different solutions with this preparation was to introduce each solution into the abomasum and to follow the secretion of acid by the pouch at 15 min intervals for 60 min. Then the solution was drained from the abomasum and the interior of the organ rinsed once with 500 ml. of saline. The conclusions were drawn from comparisons made in one experiment and not between experiments, e.g. the effect of phosphate buffers only and phosphate buffers containing fatty acid were compared on the same day.

Phosphate buffers. At pH 6·0–6·9 these stimulated acid secretion but the rate at which secretion increased was relatively slow and sometimes erratic.

Bicarbonate buffers at pH 6·1–6·7, containing 30–50 m-mole $NaHCO_3$/l., made isotonic with NaCl or phosphate and bubbled with 100% CO_2, stimulated secretion. The rate at which acid secretion increased appeared to be slightly faster than with phosphate buffer alone.

Fatty acid buffers. These were introduced at pH 5·6–7·6 and the concentration ranged from 50 to 160 m-mole/l. Solutions containing less than 160 m-mole were made approximately isotonic with phosphate buffer. The rate at which acid secretion was excited was always greater than with phosphate or bicarbonate buffers and usually the responses were more stable.

Rumen fluid. The pH of the fluid varied from 6·2–7·8 and this usually caused faster responses than phosphate or bicarbonate buffers. In three experiments the fatty acid concentration was 26–36 m-mole/l., and in one an addition of fatty acid to the fluid increased its stimulating effect.

Volume effects. In three experiments 500 ml. of phosphate buffer and 600 and 750 ml. of rumen fluid produced larger responses than 250, 400 and 500 ml. of the same solutions respectively.

pH effects. Phosphate-HCl solutions containing 50 m-mole fatty acid/l.

at pH 2·2–2·4 failed to stimulate acid secretion appreciably or caused a decrease of any residual secretion.

Figure 7 shows some of these responses.

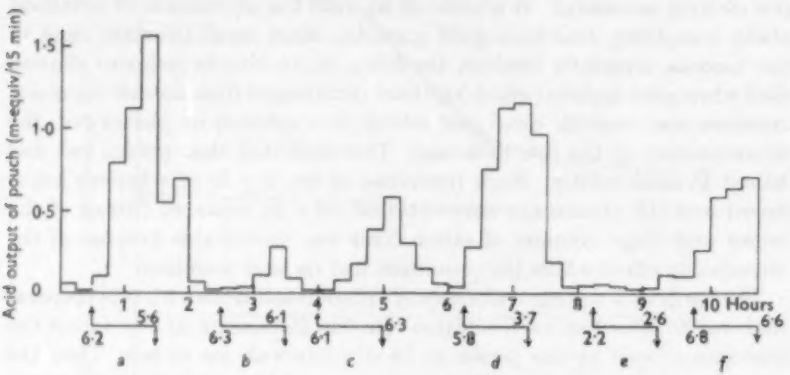

Fig. 7. A comparison of the stimulating effects of different solutions on acid secretion. *a*, phosphate + 50 mM fatty acid; *b*, phosphate buffer alone; *c*, phosphate + 30 mM $NaHCO_3$ bubbled with 100 % CO_2; *d*, phosphate + 50 mM fatty acid; *e*, phosphate-HCl + 50 mM fatty acid; *f*, rumen fluid. The numerals below and above the arrows indicate the pH values of the solutions (all volumes 450 ml.) introduced into the abomasum and drained respectively. Conscious sheep, reticulo-rumen and abomasum emptied on the morning of the experiment; abomasum 'isolated'. Maximum acid output of the pouch during normal feeding was equivalent to 1·6 m-equiv/15 min on a diet of 750 g of food/day.

Maintained responses

The previous experiments suggested that if a solution with a pH of 6–7 was introduced into the 'isolated' abomasum, acid secretion should be stimulated and maintained until the acidity of the fluid in the body of the organ increased to a value at which secretion was inhibited.

Rumen fluid and solutions containing 50–160 m-mole fatty acid/l. usually produced well-maintained responses. Acid secretion by the pouch reached a peak in 45–90 min and then showed small fluctuations before decreasing rapidly; the progressive decrease in secretion was associated with an increase in the acidity of the solution in the abomasum. The pH at which inhibition occurred was decided upon in a somewhat arbitrary way. The value taken was that at the end of the 15 min period when acid output by the pouch showed an appreciable decrease from the two preceding periods and then continued to decline. The range of pH values at which inhibition occurred was 2·0–2·6 for 160 mM fatty acid (4 experiments); 2·6 and 2·8 for phosphate buffers containing 50 mM fatty acid; 1·9 and 2·2 for rumen fluid and 2·0 for a bicarbonate buffer. These pH values are in

the same range as the contents flowing from the abomasum to the duodenum of normally fed sheep. It was therefore essential to determine the effect of abomasal fluid on acid secretion.

In two experiments the introduction of 500 ml. of abomasal fluid (pH 2·5, steam-volatile fatty acid 4 and 13 m-mole/l.) caused small but

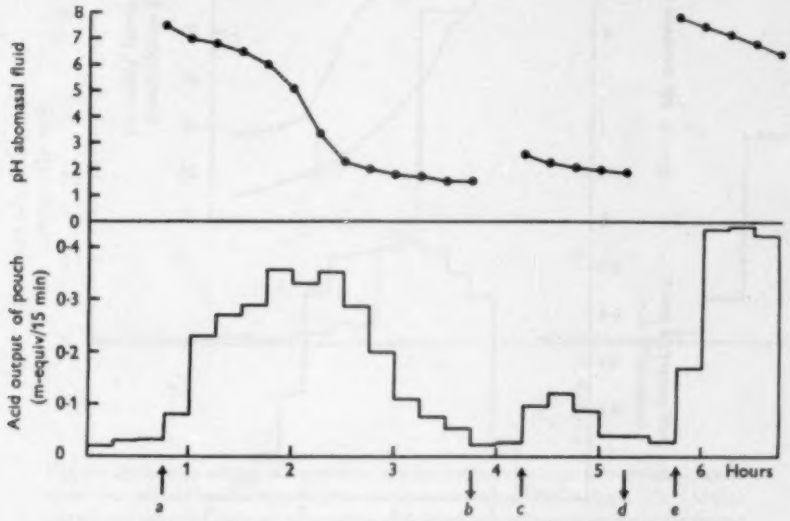

Fig. 8. Maintained secretion with rumen fluid and the stimulating effect of abomasal fluid. At *a*, 500 ml. of rumen fluid was introduced into the abomasum and drained at *b*; *c*, 500 ml. of abomasal fluid, drained at *d*; *e*, 750 ml. of rumen fluid. The concentration of fatty acid in the rumen fluid was 26 mM and in the abomasal fluid 4 mM. Note the greater rate of increase in acid secretion with 750 ml. of rumen fluid compared with 500 ml. Conscious sheep, reticulo-rumen and abomasum emptied on the morning of the experiment; abomasum 'isolated'. Maximum acid output of pouch during normal feeding was equivalent to 0·76 and 1·0 m-equiv/15 min on diets of 750 and 1250 g of food/day.

definite increases in acid secretion by the pouch; acid secretion declined at pH 1·9 in both experiments. Examples of a maintained response with rumen fluid and the stimulating effect of abomasal fluid are shown in Fig. 8. The volume of fluid drained from the abomasum at the end of the maintained responses was usually greater than that introduced, by amounts varying from 70 ml. with the phosphate-bicarbonate buffer to 360 ml. with the rumen fluids and the fatty acid buffers.

In three experiments the changes in concentration of the steam-volatile fatty acid in the abomasal fluid were followed throughout a maintained response (Fig. 9). The concentration decreased progressively and at the

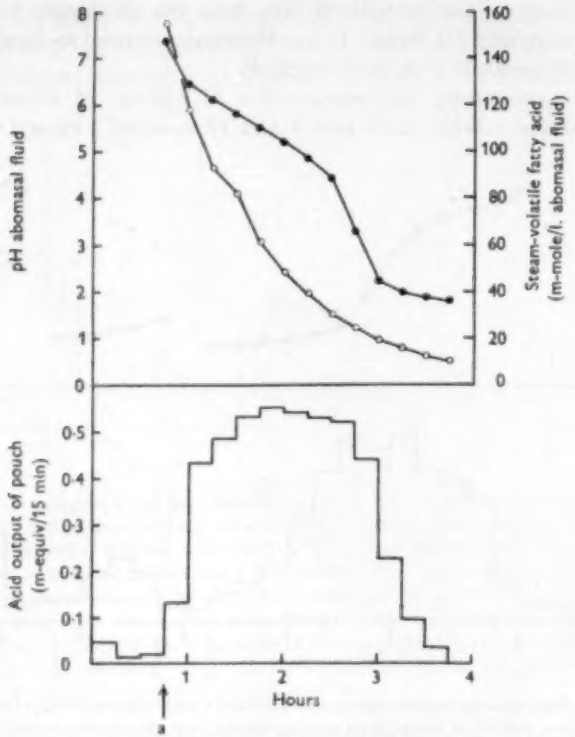

Fig. 9. An example of a maintained response with a fatty acid solution. At *a*, 500 ml. of approximately 155 mM fatty acid was introduced into the abomasum and 10 ml. samples were removed at 15 min intervals thereafter for analysis. Top panel shows the pH (●—●) and the concentration of fatty acid (○—○) in the abomasal fluid. The volume of fluid drained from the main body of the abomasum at the end of the response was 803 ml. Conscious sheep, reticulo-rumen and abomasum emptied on the morning of the experiment; abomasum 'isolated'. Maximum acid output from the pouch during normal feeding as in caption to Fig. 8.

TABLE 1. The amounts of fatty acid lost from solutions introduced into the abomasum

*Composition of solution	pH	†Amount of fatty acid (m-mole) Intro-duced	Re-covered	Lost	Final pH of soln.	Time (hr)
0·110 M phosphate + 0·050 M V.F.A.	2·4	19·8	3·6‡	16·2	2·2	1·5
0·165 M V.F.A.	5·6	65·2	8·6‡	56·6	1·8	3·0
0·110 M phosphate + 0·050 M V.F.A.	6·7	20·2	1·6‡	18·6	2·2	3·25
0·160 M V.F.A.	7·3	80·5	15·7	64·8	1·8	2·75
0·155 M V.F.A.	6·9	77·8	7·8	70·0	2·6	4·0
0·155 M V.F.A.	7·3	76·7	13·8	62·9	1·8	3·0

* Approximate fatty acid concentrations. † Amounts calculated from analysis of the solutions.
‡ Includes fatty acid recovered after rinsing abomasum; other values in this column include samples and drainings only.

inhibitory pH the values were 21·0 mM (pH 2·1), 8·7 mM (pH 2·8) and 19·2 mM (pH 2·2). The total amount of fatty acid which disappeared from the solutions in six experimental periods was calculated from the amount introduced and the amount recovered in the samples and drainings (Table 1).

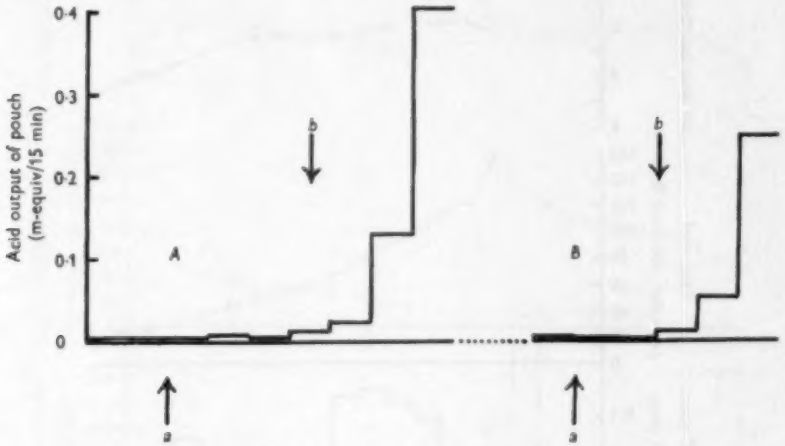

Fig. 10. Failure to excite acid secretion by introducing solutions into the abomasum when the reticulo-rumen was empty. *A*: at *a*, 400 ml. of rumen fluid (pH 6·5) was introduced into the isolated abomasum and there was no response from the pouch until 3 l. of phosphate fatty acid (pH 6·5) was introduced into the reticulo-rumen at *b*. The dotted line represents a 75 min break in the record and the rumen and abomasum were drained 45 min before the next part of the record; neither organ was rinsed. *B*: at *a*, 400 ml. of phosphate containing 100 mM fatty acid (pH 6·9) was introduced into the abomasum and again there was no acid secretion by the pouch until 3 l. of isotonic NaCl was introduced into the reticulo-rumen at *b*. Conscious sheep, reticulo-rumen emptied the night before the experiment.

Inhibition of acid secretion

Throughout the course of the work there were three experiments in which it was impossible to stimulate acid secretion by introducing solutions into the abomasum. In one of these rumen fluid (pH 6·5) and a phosphate buffer containing 100 mM fatty acid (pH 6·9) failed to stimulate within 45 and 30 min. The introduction of 3 l. of phosphate plus 120 mM fatty acid buffer (pH 6·5) and isotonic NaCl respectively into the rumen and while the solutions were still in the abomasum, resulted in the onset of a response from the pouch (Fig. 10). The animal appeared brighter and more alert after the introduction of the solutions into the rumen.

On two other occasions when maintained responses were in progress there

was a sudden and precipitous decrease in acid secretion 90 and 150 min after the solution was introduced into the abomasum; the pH values of the abomasal fluid were then 5·7 and 4·8 respectively. This form of inhibition appeared to be associated with the sudden entry into the experi-

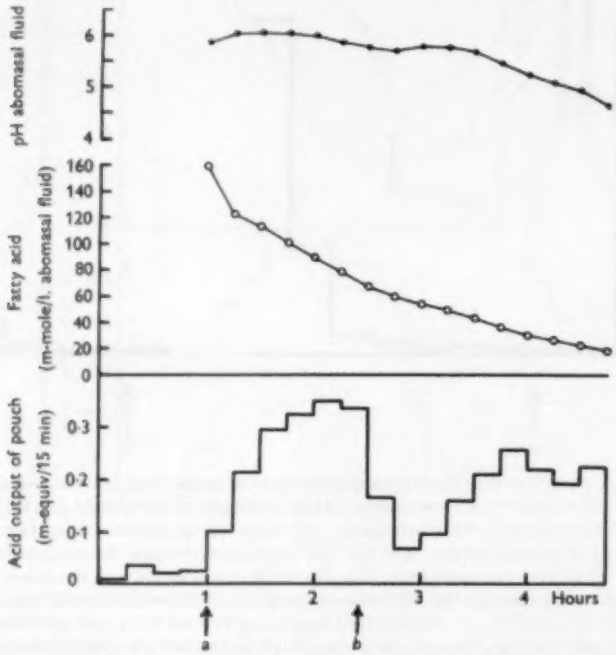

Fig. 11. Non-specific inhibition of acid secretion during a maintained response. At *a*, 500 ml. of approximately 160 mM fatty acid was introduced into the 'isolated' abomasum. At the time indicated by *b*, the routine of the experiment was interrupted by a third person entering the experimental room. Top and centre panels, pH and concentration of fatty acid respectively in the fluid in the main body of the abomasum; bottom panel, acid output of the pouch. Conscious sheep, reticulorumen emptied on the morning of the experiment. Maximum acid output of the pouch during normal feeding was equivalent to 0·48 and 1·0 m-equiv/15 min on diets of 750 and 1025 g of food/day.

mental room of a third person. Inhibition lasted for 30 min during which time the pH of the fluid in the abomasum increased slightly or decreased at a much slower rate than was usual (Fig. 11).

Inhibition from specific stimuli. The maintained response provided a means of stimulating acid secretion in a physiological way so that specific inhibitory stimuli could be studied. The excitatory stimuli employed were

rumen fluid and buffers containing 50–160 mM fatty acid at pH 6–7. Inhibitory stimuli were applied either during the rising phase of acid secretion or at the beginning of the plateau.

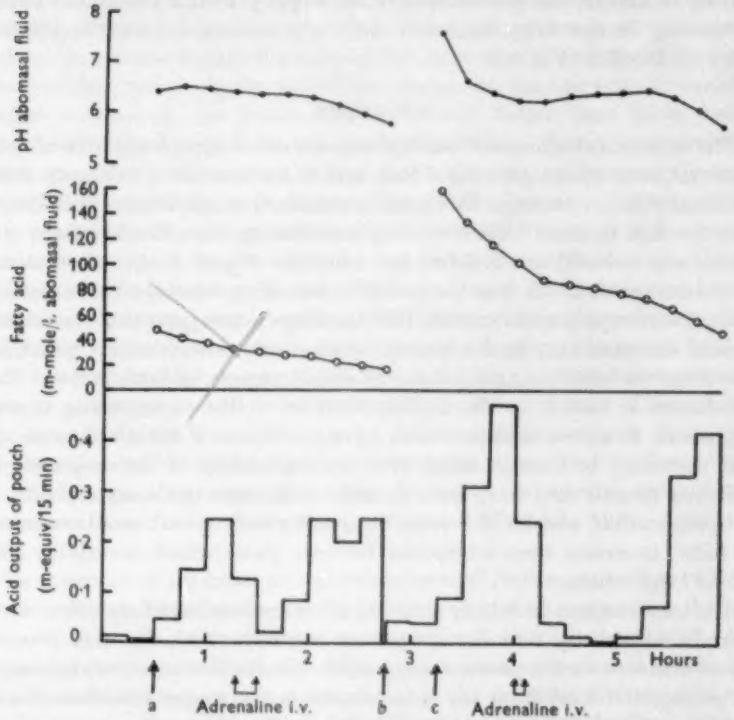

Fig. 12. Inhibition of acid secretion by adrenaline. At *a*, 750 ml. of a phosphate buffer containing 50 mM fatty acid was introduced into the 'isolated' abomasum and adrenaline 0·4 μg/kg per minute was injected intravenously during the time indicated by the arrows. At *b*, 30 min break between this and next part of the record during which the solution was drained and the abomasum rinsed with saline. At *c*, 750 ml. of 160 mM fatty acid introduced and adrenaline 0·47 μg/kg per minute was injected as indicated. Note the greater rate of rise of acid secretion with the stronger solution of fatty acid. The injections of adrenaline were made through a fine bore polythene cannula inserted into the jugular vein before the experiment. Conscious sheep, reticulo-rumen emptied on the morning of the experiment.

Infusions of isotonic NaCl-HCl (total acidity 60–62 m-equiv/l.) into the duodenum caused a profound inhibition of secretion; isotonic NaCl did not prevent the onset of a response.

Maintained responses were completely abolished by atropine 0·25 mg/kg injected intravenously and completely or partially inhibited by adrenaline

0·4 and 0·47 μg/kg/min infused intravenously over 5–10 min. The changes in pH and the concentration of fatty acid in the abomasal fluid were followed throughout the experiments when adrenaline was injected. During inhibition the pH decreased less rapidly with a phosphate buffer containing 50 mM fatty acid and definitely increased with the 160 mM fatty acid buffer (Fig. 12).

DISCUSSION

The stomach chambers of adult sheep are never completely free of food material, even after a prolonged fast, and it was necessary to empty them artificially in an attempt to obtain a standard preparation. Hill (1955) was the first to show that emptying and rinsing the reticulo-rumen and abomasum reduced or abolished the secretion of acid by the abomasum; he concluded that this was the result of removing sources of stimulation. The present experiments confirm Hill's findings but suggest that the decline in acid secretion may be due partly, at least on some occasions, to inhibition from the emptying procedure, an empty rumen, or both factors. This conclusion is based on the finding that in a few experiments it was impossible to excite acid secretion by stimulation of the abomasum. It was necessary to bear in mind that the variability of the responses to different stimuli may have been caused by changes in the excitability of the preparation, and for this reason stimuli which caused small responses or failed to excite were interposed between those which invariably produced large effects.

The experiments in which rumen fluid stimulated acid secretion when introduced directly into the abomasum are important, for they provide direct evidence for the previous suggestion that the flow into the abomasum of predigested food from the forestomach is the major stimulus of acid secretion (Hill, 1955; Ash, 1961). Moreover, if acid secretion is influenced by the amount of food material entering the abomasum it was essential to demonstrate that an increase in the volume of the inflow increased the size of the response. Rumen fluid from fasting sheep will resemble omasal fluid in that the concentration of fatty acid will be relatively low; the concentration of bicarbonate will be slightly higher and that of chloride lower than in omasal fluid (Ekman & Sperber, 1953). For the purposes of these experiments the fluid entering the abomasum under normal feeding conditions was considered to be a buffered solution of phosphate, bicarbonate and fatty acid. Predigested food from the forestomach may contain other substances, or other substances may be formed when the material is mixed with abomasal secretion, which influence the secretion of acid, but further information is required on their chemical nature before they can be investigated.

Balloon distension of the main body of the abomasum with volumes similar to those of the abomasal contents of normally fed animals clearly stimulated acid secretion. Although a direct comparison was not made in any one experiment, it did appear as though balloon distension caused a more rapid increase in secretion than either phosphate or bicarbonate buffers. It is possible that the saline-filled balloon produced a greater and more localized tension in the wall of the abomasum than an approximately similar volume of the buffer. An additional factor may have been mechanical stimulation of the mucosa by the balloon during contractions of the abomasum. It was highly improbable that the pyloric antrum was distended in any of the experiments. The decline, but not always a complete inhibition, of acid secretion, which occurred during a maintained period of distension, appeared to be due to inhibition from acid. These findings are comparable with the effects produced by distension of the pyloric antrum in the dog (Gregory, 1958). The acidity of the fluid in the main body of the abomasum at which the acid output by the pouch began to decline was usually about pH 2·0. The secondary increases in secretion during a prolonged period of distension and the delayed onset of the initial response after acid was infused into the duodenum indicate that the receptors responsible for mediating the secretion stimulated by distension show little or no adaptation. This seems to be in common with some other phenomena associated with gastric distension (Paintal, 1954; Titchen, 1958).

The influence of chemical and physicochemical factors on acid secretion were investigated most conveniently on those preparations in which it was assumed that the test solutions were confined to the abomasum. As judged by the rate at which the different solutions caused acid secretion by the pouch to increase it is concluded that the presence of fatty acid in the abomasal contents potentiated the effect of phosphate buffers. Since all the solutions were in the same pH range during the rising phase of secretion and could not flow from the abomasum the effect appears to be a true chemical one of the fatty acids (see Hunt, 1959). The aspect requiring further consideration is the importance of fatty acids in the abomasum as a factor influencing acid secretion during normal digestion and this will be related to their mode of action. Although fatty acid was absorbed from the solutions introduced into the abomasum, it would be unwise to suggest that the effect on acid secretion was mediated entirely, directly or indirectly, through an increase in the concentration of fatty acid in the circulation. A simple explanation for the effect seems unlikely, for Pennington (1952) found that *in vitro* the abomasal mucosa of sheep metabolized fatty acids remarkably well and in the following order: acetate > propionate and butyrate. In addition, Dobson & Phillipson

(1956) have shown that fatty acids increase the blood flow through rumen epithelium when fatty acid was introduced into the rumen. The possibility therefore exists that the influence of fatty acid in the abomasum on acid secretion may be partly the result of local actions which may include the increased release and/or formation of gastric hormone(s). Since the effect of fatty acid appeared to be related to the concentration in the abomasum and to whether the mechanisms are partly local ones, the significance of fatty acid in the abomasum of the normally fed animals will depend on the concentration relative to the sites of action. A criticism of the present experiments is that the mucosa of the main body of the organ and the antrum were in contact with the same concentration of fatty acid.

The stability, and hence the reliability, of the maintained responses was influenced by the concentration of fatty acid in the solution introduced into the abomasum and the external conditions of the experiment. The magnitude and the duration of the response could be controlled to some extent by adjusting the volume of the test solution and the concentration of fatty acid therein. Because of the rapid decrease in concentration of fatty acid in the test solutions through dilution and absorption it was necessary to use high concentrations in the initial solution. The maintained response does offer a way of stimulating acid secretion continuously and under relatively controlled conditions so that inhibitory stimuli may be studied.

The prepotent intra-abomasal stimulus influencing the secretion of acid was the acidity of the abomasal contents, but the threshold acidity at which secretion failed to be stimulated was not well defined. The decline in acid secretion by the pouches began at pH 1·9–2·8. The interpretation of the threshold pH in these experiments was complicated by the progressive decrease in fatty acid concentration in the test solutions. This was not the major reason for the decline in the response, because solutions containing 50 mM fatty acid at pH 2·1–2·4 also failed to stimulate. The critical pH for gastrin release in dogs is within a very narrow range and is approximately 1·5 for HCl solutions (Woodward, 1960; Woodward & Dragstedt, 1960). An important finding in the present work was that acid secretion was stimulated by abomasal contents (pH 2·5) obtained from fasting and normally fed sheep. The pH of the food material flowing from the abomasum to the duodenum during continuous acid secretion by innervated pouches is usually 2–3 and there does not appear to be any obvious correlation between the output of acid and the pH of the food material (Ash, 1961). Although acid inhibition may be a graded response to changes in the acidity of the abomasal contents, it seems most likely that the amount of acid secreted during normal feeding is influenced chiefly by the volume and possibly by the fatty acid concentration of the abomasal

contents. Support for this argument is provided by the experiments in which greater responses from the pouches were produced by larger volumes of fluid in the abomasum (Figs. 2 and 8), solutions containing fatty acid (Figs. 6 and 7) and different concentrations of fatty acid (Fig. 6).

In some of the experiments an increase in the pH of the abomasal contents was observed during the initial stages of acid secretion and also when acid secretion was inhibited. There is evidence that when acid secretion is abolished the residual secretion contains bicarbonate (Ash, 1961); this factor plus the normally alkaline secretion of the antrum (Ash, 1959*c*) would tend to increase the pH of the abomasal contents slightly during a complete inhibition of acid secretion. Other mechanisms contributing to the pH changes may be the rapid absorption of free fatty acid (Danielli, Hitchcock, Marshall & Phillipson, 1945) and the exchanges of CO_2 and bicarbonate (Masson & Phillipson, 1951; R. W. Ash and A. Dobson, unpublished observations) which have been demonstrated in the rumen. An increase in the pH of the abomasal contents can be expected provided similar mechanisms occur in the abomasum when fatty acid is absorbed and at a greater rate than acid secretion is stimulated.

The outputs of acid by the pouches when stimuli were applied solely to the abomasum were usually less than the maxima observed in the same sheep during feeding experiments. Such a comparison is not really valid because there may have been differences in the absolute or relative strengths of the stimuli under the two entirely different sets of experimental conditions. It is difficult to know whether the animal with an empty reticulo-rumen is partly inhibited or whether there is direct excitation of acid secretion from the forestomach of normally fed sheep. The experiments reported in this paper do not preclude the excitation of acid secretion from the forestomach and duodenum, but it is quite clear that acid secretion by the abomasum can be controlled adequately through the conditions within the abomasum.

SUMMARY

1. Preparations and methods are described which allow the study of intra-abomasal factors on acid secretion by innervated abomasal pouches.

2. Distension of the main body of the abomasum with a saline-filled balloon stimulated acid secretion; inhibition of secretion occurred when the acidity of the free fluid in the abomasum increased to about pH 2·0.

3. The mechanisms responsible for mediating the secretion stimulated by distension showed little or no adaptation.

4. The introduction of rumen fluid and buffered solutions at pH 5–7 directly into the abomasum stimulated acid secretion. Secretion was usually well maintained until the acidity of the fluid in the abomasum

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increased to pH 1·9–2·8. Large volumes of fluid produced greater responses than small ones.

5. The presence of fatty acid (acetic:propionic:butyric, 70:20:10, 25–50 mM) and increased concentrations (50–160 mM) of these metabolites in the solution potentiated the responses.

6. Fatty acid was absorbed from the solutions introduced into the abomasum.

7. Phosphate-HCl solutions (pH 2·1–2·4) containing 50 mM fatty acid failed to stimulate acid secretion appreciably when introduced into the abomasum. Abomasal fluid (pH 2·5) from fasting and normally fed sheep evoked a small but definite response.

8. Acid secretion was inhibited by psychic stimuli, the introduction of HCl into the duodenum and by intravenous injections of adrenaline and atropine.

9. The possible role of stimuli acting in the abomasum on acid secretion during normal digestion in the sheep is discussed.

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REFERENCES

Ash, R. W. (1959*a*). Inhibition and excitation of reticulo-rumen contractions following the introduction of acids into the rumen and abomasum. *J. Physiol.* **147**, 58–73.

Ash, R. W. (1959*b*). Acid secretion by the abomasum and its relation to the acidity of the abomasal contents. *J. Physiol.* **149**, 72–73*P*.

Ash, R. W. (1959*c*). Abomasal secretion in sheep: changes in ionic composition with rate of flow. *16th Int. Veterinary Congress. Madrid.* Communication (1*a*) 6.

Ash, R. W. (1961). Acid secretion by the abomasum and its relation to the flow of food material in the sheep. *J. Physiol.* **156**, 93–111.

Benzie, D. & Phillipson, A. T. (1957). *The Alimentary Tract of the Ruminant*, p. 24. Edinburgh: Oliver and Boyd.

Danielli, J. F., Hitchcock, M. W. S., Marshall, R. A. & Phillipson, A. T. (1945). The mechanism of absorption from the rumen as exemplified by the behaviour of acetic, propionic and butyric acids. *J. exp. Biol.* **22**, 75–84.

Dobson, A. & Phillipson, A. T. (1956). The influence of the contents of the rumen and adrenaline upon its blood supply. *J. Physiol.* **133**, 76–77*P*.

Ekman, J. & Sperber, I. (1953). The distribution of concentrations of bicarbonate (including carbon dioxide) and chloride in the omasum of cows. *Kungl. Lantbrukshögskolans Annaler.* **19**, 227–231.

Gregory, R. A. (1958). Gastric secretory responses after portal venous ligation. *J. Physiol.* **144**, 123–137.

Hill, K. J. (1955). Continuous gastric secretion in the ruminant. *Quart. J. exp. Physiol.* **40**, 32–39.

Hunt, J. N. (1959). Gastric emptying and secretion in man. *Physiol. Rev.* **39**, 491–533.

Masson, M. J. & Phillipson, A. T. (1951). The absorption of acetate, propionate and butyrate from the rumen of sheep. *J. Physiol.* **113**, 189–206.

Masson, M. J. & Phillipson, A. T. (1952). The composition of the digesta leaving the abomasum of sheep. *J. Physiol.* **116**, 98–111.

OYAERT, W. (1955). *Studie van de wyziging der minerale en stikstofhondende fractie van het voldertydenes de passage doorhen de voormagen.* Ph.D. Thesis. University of Ghent.

PAINTAL, A. S. (1954). A study of gastric stretch receptors. Their role in the peripheral mechanism of satiation of hunger and thirst. *J. Physiol.* **126**, 255–270.

PENNINGTON, R. J. (1952). The metabolism of short chain fatty acids in the sheep. 1. Fatty acid utilization and ketone body production by rumen epithelium and other tissues. *Biochem. J.* **51**, 251–258.

TITCHEN, D. A. (1958). Reflex stimulation and inhibition of reticulum contractions in the ruminant stomach. *J. Physiol.* **141**, 1–21.

WOODWARD, E. R. (1960). The role of the gastric antrum in the regulation of gastric secretion. *Gastroenterology.* **38**, 7–14.

WOODWARD, E. R. & DRAGSTEDT, L. R. (1960). Role of the pyloric antrum in regulation of gastric secretion. *Physiol. Rev.* **40**, 490–504.

PROCEEDINGS OF
THE PHYSIOLOGICAL SOCIETY

UNIVERSITY COLLEGE LONDON MEETING

24–25 *March* 1961

The chemical nature of the menstrual stimulant components *A*, *B*1 and *B*2

By H. J. Clitheroe and V. R. Pickles. *Department of Physiology, University of Sheffield*

The methods by which three lipoid stimulants or groups of stimulants, referred to as components *A*, *B* and *C*, may be prepared from human menstrual fluid have been described in detail (Clitheroe & Pickles, 1961). Chromatography of larger quantities of lipid has now shown that component *B* consists of two substances, referred to as *B*1 (the more polar) and *B*2 (the less polar).

Component *A* is thought to include a γ-lactone with an additional polar group, probably –OH, upon which its uterine muscle-stimulant activity partly depends. *B*1 and *B*2 are probably long-chain fatty acids.

The demonstration consists of a display of some of the evidence upon which these provisional conclusions are based.

We wish to thank Dr R. Brettle for directing infra-red and other analyses in the Sheffield University Department of Chemistry.

REFERENCE

Clitheroe, H. J. & Pickles, V. R. (1961). *J. Physiol.* **156**, 225–237.

Identification of acetylcholine in the silk gland of the caterpillar of *Arctia caja* (L.).

By J. Morley and M. Schachter. *Department of Physiology, University College London*

It has been shown recently that high concentrations of active esters of choline are present in certain insects. Thus, ACh has been identified in hornet venom in amounts varying from 10 to 50 mg/g dry venom sac (Bhoola, Calle & Schachter, 1960). Another cholinester, probably $\beta_1\beta$-dimethylacrylylcholine (DMAC) is secreted by the prothoracic gland of the Garden Tiger Moth, *Arctia caja* (Bisset, Frazer, Rothschild & Schachter, 1960).

On examination of the tissues of the larva of *A. caja*, ACh was identified by parallel bio-assay and by paper chromatography in the hypertrophied silk glands of the final larval instar. The amount of ACh is approximately 4 mg/g freeze-dried gland. The 'silk' (cocoon) secreted by the larva before the pupation also contains ACh, approximately 300μg/g.

Acid extracts of the freeze-dried silk glands of *A. caja* larvae tested on the isolated guinea-pig ileum, frog rectus abdominis muscle, leech body wall and cat blood pressure, yield ACh concentrations varying from 3 to 4·5 mg/g dry gland. The activity is abolished either by atropine (10^{-8} g/ml.) or by D-tubocurarine (10^{-6} g/ml.).

Paper chromatograms of ethyl alcohol extracts of 0·5–2·0 mg dry glands in several solvents (*n*-propanol-formic acid-water, 8:1:1; *n*-propanol-water, 9:1; *n*-butanol-ethanol-acetic acid-water, 8:2:3) yield on elution a substance corresponding with ACh in R_F value, iodine reaction, and in pharmacological activity when tested in parallel on the guinea-pig ileum and frog rectus muscle.

ACh is the only pharmacologically active substance detected by these tests in *A. caja* larvae. DMAC, which is present in high concentrations in the prothoracic gland of the adult moth, is not detectable in the larva.

Treatment of the frog rectus abdominis muscle with acetone (0·5–1·0 %) considerably increased its sensitivity to ACh and such a preparation is very useful for ACh assay. By adding acetone to the eserinized (5×10^{-6} g/ml.) Ringer's solution to the above concentration, the sensitivity of the rectus muscle is always increased, usually five to ten-fold.

REFERENCES

Bhoola, K. D., Calle, J. & Schachter, M. (1960). *J. Physiol.* **151**, 35–36*P*.

Bisset, G. W., Frazer, J. F. D., Rothschild, Miriam & Schachter M. (1960). *Proc. Roy. Soc.* B, **152**, 255.

An analogue model of the heart

By J. E. Malcolm (introduced by M. Grace Eggleton). *Postgraduate Medical School of London, Ducane Road, London, W.* 12

A comparison was drawn between the action of the human heart and that of a reflex klystron, by Malcolm (1960). In order to develop the theory further, a model (Fig. 1) of the heart has been built, to which the following description applies.

Fluid which may be water, saline solution or blood, is drawn from the reservoir (*A*) by the 'atrium' (*B*), a collapsible tube surrounded by a rigid tube of greater size. The space between the two tubes contains air

which is alternately compressed and rarefied, so imparting a wave to the fluid. The latter passes through a 'mitral valve' (*C*) and enters the 'left ventricle' (*D*) where it receives a second pressure wave, delivered in a

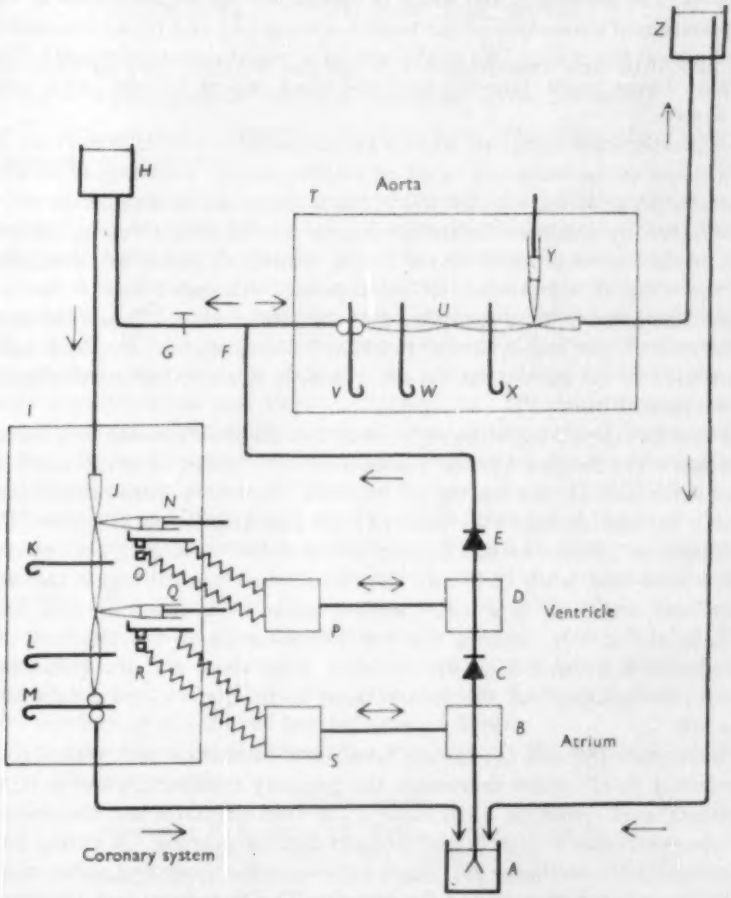

Fig. 1. Diagram to show the relationship of the components of the analogue model of the heart.

similar manner. An 'aortic valve' (*E*) determines flow in the direction of the T-piece (*F*) where part of the fluid, controlled in amount by a screw valve (*G*), is diverted to rise to a height of 30 cm into the reservoir (*H*).

From here it descends to an 'oscillator' (*I*) formed by a latex rubber tube (*J*), 0·7 cm in diameter and 97 cm long, arranged horizontally upon

a small platform. The outflow is led by a rigid tube to the reservoir (*A*) and provided the outlet of this tube is adjusted to a level slightly below that of the platform, the elastic tube (*J*) will oscillate in stationary wave motion. The frequency and mode of oscillation can be controlled by the application of extensions of the torsion springs (*K*) and (*L*) to the sites of anti-nodes: the spring (*M*) to the site of a pseudo-stationary node. Two sets of waves result, long (75 cm) and short (about 1·5 cm), with loops as shown.

A quarter wave matching stub (*N*) is connected to a node of the tube (*J*) and closed at the other end by an adjustable piston. Pulsation of the stub operates an electrical contact (*O*) which actuates the pulse generator (*P*). The latter, by means of electrical relays and solenoid gas valves, controls the production of pressure waves in the 'ventricle', power being supplied in the form of compressed air subsequently exhausted into a vacuum tank. The quarter wave matching stub (*Q*), the contact (*R*) and the wave generator (*S*) perform a similar function for the 'atrium' the stub being connected to the tube (*J*) at the site of a node situated half a wave-length down stream from (*N*).

Fluid flowing through the right-hand branch of (*F*) passes to a second oscillator (*T*) formed by the elastic tube (*U*) which is compressed by the extension of the spring (*V*) at the site of a pseudo-stationary node: by the springs (*W*) and (*X*) at the sites of anti-nodes. This oscillator is similar to the former but is fitted with only one quarter wave matching stub (*Y*) and acts in reverse, converting a pulsatile flow into constant flow: the outflow leads to a reservoir (*Z*), fixed at a height of 1 m, whence the overflow descends to the reservoir (*A*). The latter represents both the systemic great veins and the pulmonary veins: the right side of the 'heart' must be imagined as lying parallel to the left.

Reservoirs (*H*) and (*Z*) having been filled, oscillation commences spontaneously in (*I*) which represents the coronary system. Pulsation in the 'atrium' and 'ventricle' then follows, the fluid circulates and the pressure in the reservoirs is maintained without further priming. A strong pulse develops in the oscillator (*T*), which represents the ascending aorta, especially pronounced at resonant frequencies. The frequency and phasing of pulsation of the two chambers of the 'heart' are determined by tuning the oscillators.

The model shows that abnormalities of cardiac rhythm are related fundamentally to the modes of oscillation of fluid-filled elastic systems. It may be employed to study the effects of fatigue upon the wall of a pulsating elastic tube and the production of sound waves by the fluid flowing therein. The behaviour of the model suggests that a pulse at certain

anatomical sites, for example, the Circle of Willis, may develop from the static pressure of the blood in the great vessels.

REFERENCE

MALCOLM, J. E. (1960). *Nature, Lond.*, **186**, 723.

An electron-microscopic survey of the structure and composition of the developing optic nerve in *Xenopus laevis* (Daudin)

By R. M. GAZE and A. PETERS. *Departments of Physiology and Anatomy, University of Edinburgh*

The optic nerve of *Xenopus*, in common with that of other anurans, of urodeles and of some fishes, will regenerate after section (Gaze, 1959). Maturana (1959) has shown, by use of the electron microscope, that the optic nerve of *Rana pipiens* contains about 470,000 non-myelinated fibres, or some thirty times as many as were previously reported (Bruesch & Arey, 1943). An investigation of fibre composition has now been carried out on the optic nerve of *Xenopus*, at various stages of development, using phase-contrast and electron microscopy. The first appearance of visual responses (optokinetic reactions to a rotating striped drum) occurs in the tadpole of stage 49, and at the same stage myelin sheaths are first found in the optic nerve. At stage 49 the nerve contains 2000–5000 non-myelinated fibres. From stage 49 onwards the number of myelinated and non-myelinated fibres increases until at stage 57, just before metamorphosis, there are approximately 1000 myelinated fibres and 22,000 non-myelinated ones. Throughout development, from stage 49 up to the adult, the distribution of myelinated fibre diameters shows a single peak, at 1·5 μ. The distribution of non-myelinated fibre diameters also has a single peak, between 0·1 and 0·4 μ. The optic nerve of adult *Xenopus* contains some 6000 myelinated and 33,000 non-myelinated fibres.

This demonstration illustrates the structure of the nerve at various stages of development.

REFERENCES

BRUESCH, S. R. & AREY, L. B. (1942). *J. comp. Neurol.* **77**, 631.
GAZE, R. M. (1959). *Quart. J. exp. Physiol.* **44**, 290.
MATURANA, H. R. (1959). *Nature, Lond.*, **183**, 1406.

A micromanipulator

By A. F. HUXLEY. *Department of Physiology, University College London*

The pair of micromanipulators being demonstrated was designed by the author while at the Department of Physiology, Cambridge University, and built in the workshops of the Engineering Laboratory, Cambridge University.

The principal features of the instruments are the following:

(1) Fine movements in three directions at right angles are made by operating large-diameter micrometer heads. Movements in the three principal directions can therefore be made independently.

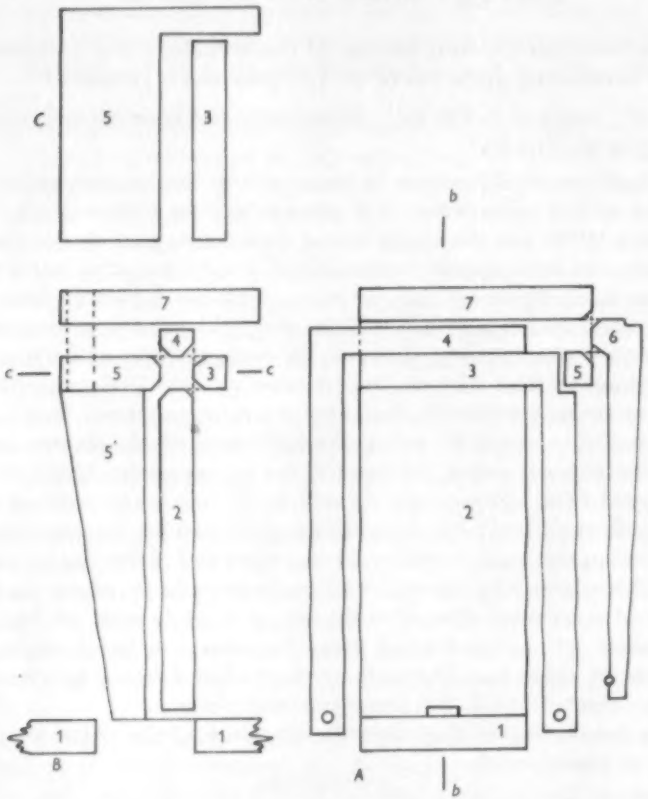

Fig. 1. Diagrams showing means by which the fine motions are superposed. *A*, left-hand manipulator seen from the front; *B*, section along *b–b*; *C*, section along *c–c*. 1, base; 2, fixed upright member; 3, member producing fore and aft motion; 4, member producing up and down motion; 5, member undergoing motions imparted by 3 and 4, prevented from rocking in the fore and aft plane by an extension 5′ connected to 1 by a flexible strip; 6, member producing right and left motion; 7, top plate undergoing all three motions, prevented from pivoting about its hinge with piece 6 by the strip connecting it to piece 5 shown dotted at the left-hand side in *A* and *B*. Circles represent balls to which the thrusts from the corresponding micrometer heads are imparted by push-rods against spring loading. The crossed strips forming the hinges are indicated by broken lines; there are two vertical and two horizontal strips in each of the five hinges.

(2) The micrometers are all mounted on the base of the instrument, so that the weight of the operator's hand is not applied to any of the motions of the instrument.

(3) The travel of each micrometer is transferred to the top plate of the instrument (7, Fig. 1), through a push-rod and a 10:1 reduction lever, so that 1 division on the micrometer drum represents 0·2 μ movement of the plate.

(4) The pivots of all the levers are crossed strip spring hinges so that backlash, friction and lubrication difficulties are completely avoided.

(5) The instruments are of very rigid construction.

(6) The performance of the instrument does not depend on high precision workmanship in any part except the micrometer heads themselves.

The arrangement by which the three motions are superposed is illustrated in Fig. 1.

Coarse movements are provided by three rack-and-pinion motions mounted on top of one another on the plate 7 which is controlled by the micrometers.

The fine motions are along arcs of circles, not straight lines, but the deviations from straight-line motion are extremely small within the field of a microscope. Backlash is not detectable under the conditions in which the instrument has so far been used (magnifications up to 1000 ×).

The potential level at the surface of the cerebral cortex of the rat and its relation to the cortical activity evoked by sensory stimulation

By O. C. J. Lippold, J. W. T. Redfearn and Lynn J. Winton. *Department of Physiology, University College London*

The sensory evoked potential which can be recorded from the cortex of the rat is of extremely variable size and form. This is particularly true when the experimental animal is in good condition, is lightly anaesthetized, and the recording is made through the intact dura exposed by a 0·4 mm diam. trephine hole in the skull. There are spontaneous changes in the size of the potential, which ranges from a maximum peak voltage of 5 mV, to the absence of any measurable change. If precautions are taken to keep the cortical surface at body temperature, the evoked potentials are usually very small and inconstant (50–100 μV peak amplitude), while cooling the surface by 2–3° C is associated with evoked potentials of constant shape having large negative waves (3–4 mV amplitude).

It is well known that fluctuations in the potential level of the cortex occur; for example, the surface becomes positive relative to the rest of the

body during maintained sensory stimulation. We find that the form, size and polarity of the evoked potential are related to the pre-existing surface potential of the cortex. This seems to be true whether the changes in potential are spontaneous, for example, during the course of light anaesthesia with urethane or avertin, or whether these potential changes are produced experimentally. The methods we have employed in order to bring about changes in the potential level have been repetitive sensory stimulation, local application of K^+ (Krebs's solution containing 10–15 mM

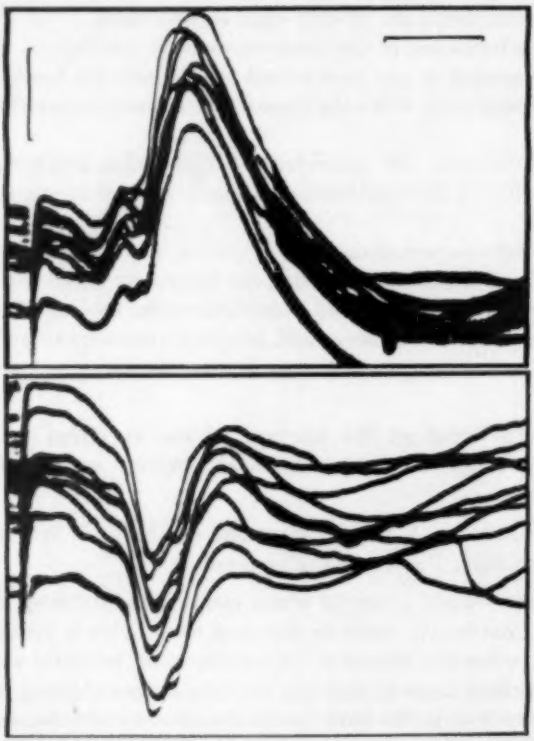

Fig. 1. Evoked potentials from the surface of the sensory cortex of the lightly anaesthetized rat (avertin, 2½%, 0·9 ml./100 g body wt. I.P.). Ten consecutive stimuli at 5 sec intervals given to sensory nerve in forelimb. Lower record shows the effect of surface-negative polarization (non-polarizable bristle on cortical surface, indifferent electrode in subcutaneous tissue of the neck, total current flow = 3 μA). Upper record is of surface-positive polarization. In both records a downward deflexion denotes that the recording micro-electrode is positive with respect to an indifferent electrode. Time bar = 10 msec. Voltage calibration = 1 mV.

K^+), local cooling and warming of the cortex and electrical polarization (see Fig. 1).

When the cortical surface undergoes a positive fluctuation, the evoked potential becomes larger and the second wave is large and negative (3–4 mV). A negative swing in the d.c. level is associated with a small or absent evoked potential and often the second wave is positive.

Working models of a semicircular canal

By D. H. Thompson and W. J. Tonkins. *R.A.F. Institute of Aviation Medicine, Farnborough, Hants*

From the data provided by van Egmond, Groen & Jongkees (1949) and criteria of similarity derived by K. E. Spells (private communication) enlarged models can be constructed which are dynamically similar to the living mechanism. In each canal model the ampulla-cupula system is replaced by an accurately machined cylinder and piston, the vestibule

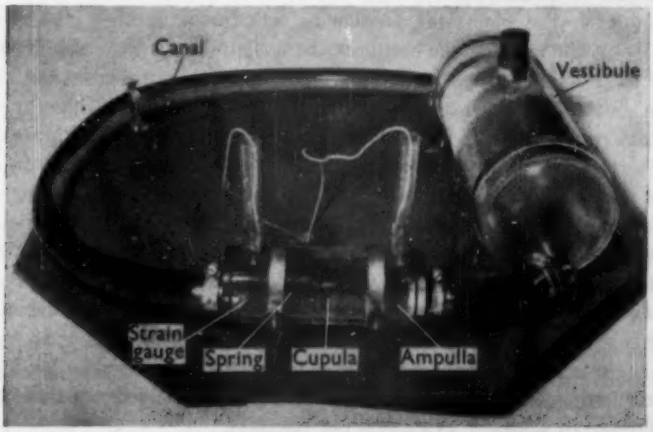

Fig. 1

by a larger cylinder, and the canal by a ring-shaped tube. The cupula restoring force is provided by two springs situated one on each side of the piston. These springs are in turn attached to strain gauges which serve to measure piston displacement. The models are filled with a liquid of suitable viscosity representing the endolymph.

In the first model, Fig. 1, the strain-gauge output is displayed on a galvanometer. In the second model the amplified output controls the

velocity of a servomotor mounted co-axially with the canal. The motor is caused to fly back when a pre-set deviation is reached. A plane mirror, which represents the eye, is driven by this motor. A beam of light reflected from this mirror allows its motion to be displayed and the whole system can be used to demonstrate nystagmus when the canal is rotated.

REFERENCE

VAN EGMOND, A. A. J., GROEN, J. J. & JONGKEES, L. B. W. (1949). *J. Physiol.* **110**, 1–17.

Transverse impedance measurement of striated muscle

By P. FATT. *Department of Biophysics, University College London*

The impedance of a flat muscle (frog sartorius or rat diaphragm) is measured by the application of a transverse electric field in the frequency range 1·5 to 130,000 c/s. Electrical contact is made with each side of the muscle through a saline-filled compartment separated from the muscle by a sheet of insulating material with a circular hole of 0·07 cm^2 area. Contact with the electrical measuring apparatus is then made by Pt black-Pt on the walls of the compartment. The area of Pt is about 20 times that of the hole, to reduce the proportion of the total impedance contributed by the electrolyte-Pt interface at the lower frequencies. Spacing of the electrical contacts with the muscle is controlled by a micrometer screw. The electrical measuring apparatus consists of a direct comparison bridge with a frequency-selective amplifier as detector for frequencies below 30 kc/s. The peak-to-peak voltage applied between the terminals of the electrode assembly at bridge balance is 15 mV. This is below the voltage at which variation of impedance with applied voltage becomes appreciable. The sensitivity of the detector and accuracy of the known arms of the bridge is such as to allow measurement of absolute impedance to be made to an accuracy better than 1 part in 1000 (for frequencies below 30 kc/s.) Measurements of complex impedance are obtained at frequencies differing by the ratio of about 2·2 (3 steps per decade).

Results obtained in a frog sartorius muscle soaked in solutions of different conductivity (NaCl replaced by sucrose) are illustrated. Specific impedance (in Ω cm) for the muscle is plotted in the complex plane, the equivalent series resistance and reactance being plotted as abscissa and ordinate, respectively. A circular curve with centre below the abscissal axis fits the points for higher frequencies, as described by Bozler & Cole (1935). This impedance locus is attributed to current flowing through the muscle fibres, traversing the surface membrane with a capacity of about 2 $\mu F/cm^2$ and the myoplasm with a resistivity of about 200 Ω cm.

A circular curve also fits the points for lower frequencies, as described for frog thigh muscle in Ringer's solution by Schwan (1954). While the magnitude of dispersion (taken as the difference in the intercepts of the circular

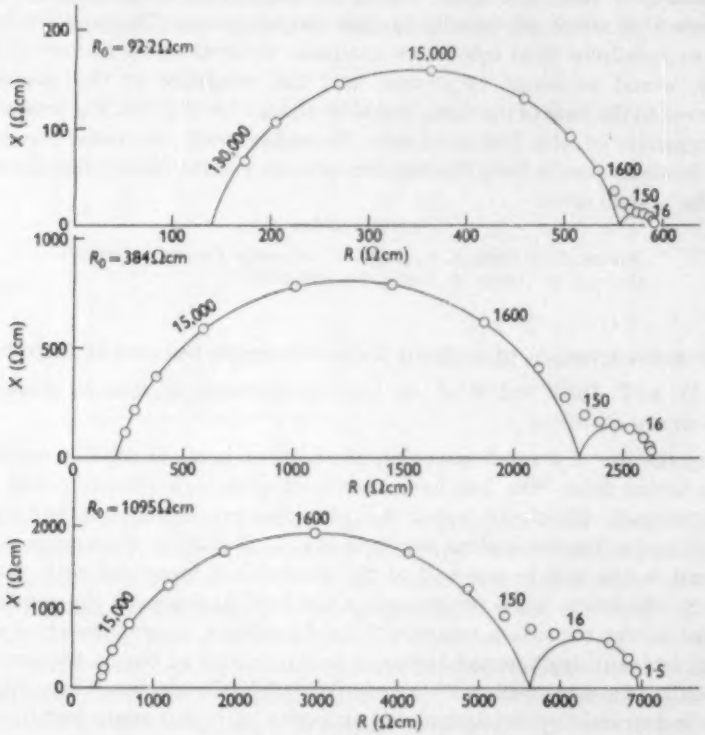

Fig. 1. Plots of transverse impedance measurements on a frog sartorius muscle in solutions of different resistivities (R_0). Impedance of the electrodes in series with the muscle has been subtracted. Impedance is represented as equivalent series resistance (abscissa) and capacitative reactance (ordinate) reduced to unit geometry. Frequencies in c/s are given for some of the plotted data. Circular curves are drawn in to show dispersions at high and at low frequencies. Upper plot gives data obtained with the muscle in Ringer's solution; for the other two NaCl was reduced by replacement with sucrose. Temperature was 17·1–17·6° C.

curve on the abscissal axis) is small for low frequencies compared to that for high, so that a high degree of accuracy of measurement is required to observe the low-frequency behaviour, this is made more prominent by decreasing the conductivity of the extracellular fluid as shown in the figure.

Results previously obtained with micro-electrodes rule out the possibility that the current flow producing the low-frequency dispersion passes through the myoplasm. It is tentatively proposed that the low-frequency dispersion is due to current flowing through separate channels in the muscle fibre which electrically by-pass the myoplasm. The capacity from the extracellular fluid into these channels, referred to the surface of the fibre, would be about 70 μF/cm^2, and the resistance of the channels, referred to the bulk of the fibre, would be about 15,000 Ω cm. The increased prominence of the low-frequency dispersion with decreased external conductivity results from the relative constancy of the electrical properties of the muscle fibre.

REFERENCES

Bozler, E. & Cole, K. S. (1935). *J. cell. comp. Physiol.* **6**, 229–241.
Schwan, H. (1954). *Z. Naturf.* **9B**, 245–251.

The measurement of sodium fluxes in single oocytes of *Bufo bufo*

By D. A. T. Dick and E. J. A. Lea. *Department of Human Anatomy, University of Oxford*

By the use of a small sandwich of phosphor in a scintillation counter, β-radiation from ^{24}Na has been measured with high efficiency and low background. Plastic phosphor NE 102 (Nuclear Enterprises Ltd.) was machined to the dimensions shown in Fig. 1. Radiation from a single cell placed in the well in one half of the sandwich is measured with almost 100% efficiency, while by designing the half-thickness of the sandwich equal to the maximum range of ^{24}Na β-particles, approximately 6 mm, a minimum of background radiation is intercepted so that a background of only 16 counts/min is obtained. With ^{24}NaCl solution (Code SGS 1, Radiochemical Centre, Amersham) activities up to 6·0 counts/min/p-mole Na have been recorded on the counter, giving an activity of up to 300 counts/sec/cell. By use of a braking pipette (Holter, 1943) the cell can be recovered after measurement so that repeated counts can be made on the same cell.

Individual oocytes 500–900 μ in diameter are obtained by treating the ovaries of young females for 1 hr at 30° C with 0·5% trypsin in calcium-free Ringer's solution adjusted to pH 8·0. Subsequent Na and K concentrations measured on groups of seven oocytes by flame photometry averaged 20 and 105 mM/l. cells for the first 20 hr, and 30 and 100 mM/l. cells between 20 and 30 hr after trypsinization. Water content averaged 72%. Na fluxes were measured by immersion in phosphate-buffered Ringer's solution containing ^{24}NaCl. During uptake of ^{24}Na, activity is measured after five washes in inactive solution and the time occupied by this and by the

actual counting necessitates an interruption in the uptake of activity during which exponential loss is assumed. Efflux is measured in inactive Ringer's solution. In a typical experiment influx was 1·65 p-mole/cm^2.sec and the efflux rate constant 0·00109 min^{-1}. These flux rates may be converted to permeability coefficients assuming that flux is proportional to

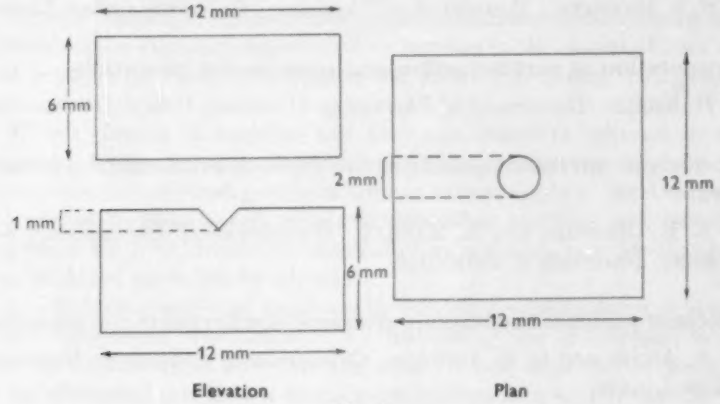

Fig. 1

concentration, giving k_{in} $1{\cdot}5 \times 10^{-4}$ μ/sec and k_{out} 23×10^{-4} μ/sec. These are comparable with those obtained by Hodgkin & Horowicz (1959) on single frog muscle fibres, 2·9 and 38×10^{-4} μ/sec, respectively, but much lower than those of Abelson & Duryee (1949) in frog oocytes, 61·6 and 1700×10^{-4} μ/sec. It may be noted that Abelson & Duryee obtained oocytes by dissection and found an internal Na concentration of 39 mM/l. cells, so that the state of the cells was probably different. Efflux appears to be strictly exponential between 1 and 10 hr, but a fast component is often present during the first hour while between 10 and 30 hr the loss rate was lower. However, calculation suggests that only 40–50 % of the cell Na is replaceable by labelled sodium.

The expenses of this work were met by a grant from the Medical Research Council.

REFERENCES

Abelson, P. H. & Duryee, W. R. (1949). *Biol. Bull., Woods Hole*, **96**, 205.
Hodgkin, A. L. & Horowicz, P. (1959). *J. Physiol.* **145**, 205.
Holter, H. (1943). *C.R. Lab. Carlsberg*, ser. chim. **24**, 399.

An instrument trolley for use in mammalian physiology

By D. R. Bacon, M. de Burgh Daly and Mary J. Scott. *Department of Physiology, St Bartholomew's Hospital Medical College, London, E.C.* 1

Responses to acetylcholine in aneural segments of frog muscle

By B. Katz and R. Miledi. *Department of Biophysics, University College London*

The membrane constants of denervated skeletal muscle

By S. J. Hubbard. *Department of Physiology, University College London*

Computation of cardiac action and pace-maker potentials

By D. Noble. *Department of Physiology, University College London*

An electron microscope study of two types of avian skeletal muscle fibre

By B. L. Ginsborg and B. Mackay. *Departments of Pharmacology and Anatomy, University of Edinburgh*

Effects of peripheral stimulation on unit discharges in the thalamus

By A. Angel and G. D. Dawson. *Department of Physiology, University College London*

A fluorophotometer for measuring the concentration of fluorescein in the tissues of the eye

By D. M. Maurice. *Ophthalmological Research Unit, Institute of Ophthalmology, Judd Street, London, W.C.* 1

A frequency integrator for recording nerve discharges

By R. A. Webster. *Department of Pharmacology, University College London*

Cholinergic potentiation of the response to gastrin

By M. I. Grossman. *The Physiological Laboratory, University of Liverpool*

Gastrin prepared by the method of Gregory & Tracy (1960, 1961) was given subcutaneously every 15 min to dogs with totally extrinsically denervated fundic pouches (Gregory, 1958). When the rate of acid secretion reached a plateau, the dose of gastrin was doubled and this procedure was repeated until no further rise in acid secretion occurred. In three dogs the maximal rate of acid secretion attained varied from 12 to 35% of the maximum achieved with histamine stimulation under similar test conditions. When Mechothane (urethane of beta-methyl choline) was given in a dose of 0·2 mg subcutaneously every 15 min for 3 hr before gastrin

and then continued throughout the remainder of the test, the maximum rate of secretion in response to gastrin was 2–8 times the level achieved without Mechothane. The maximum rate of acid secretion in response to histamine with Mechothane was 1·2–1·4 times the level without Mechothane. The maxima with gastrin plus Mechothane were 60–78 % of the maxima with histamine plus Mechothane.

Superimposition of submaximal gastrin stimulation upon a background of Mechothane stimulation produced an increase in the output of both acid and pepsin which was sustained for the entire 3 hr period during which gastrin was given.

After a plateau of secretion had been established in response to submaximal doses of gastrin every 15 min, 1·2 mg atropine sulphate was given intravenously and gastrin injections were continued. Acid secretion fell almost to basal levels within 30 min after atropine and remained depressed for 2 hr. Secretion stimulated at a similar rate by histamine was inhibited much less by atropine.

An effect similar to that produced by providing a background of Mechothane stimulation was achieved by distending the denervated fundic pouches with 80 ml. of 0·9 % NaCl solution. In four dogs the acid response of the distended pouch to a single subcutaneous injection of gastrin was 2·1–5·8 times that of the undistended pouch. Pepsin output in response to gastrin injection was also increased by distension of the pouch.

It is concluded that (*a*) cholinergic stimulation potentiates the action of gastrin more than the action of histamine, (*b*) against a background of cholinergic stimulation gastrin can produce a sustained increase in pepsin output, and (*c*) distension of an extrinsically denervated fundic pouch results in potentiated acid and pepsin responses to injected gastrin. The latter effect is assumed to be mediated by a local cholinergic reflex which probably operates when a meal is eaten.

REFERENCES

GREGORY, R. A. (1958). *J. Physiol.* **144**, 123.
GREGORY, R. A. & TRACY, H. J. (1960). *J. Physiol.* **154**, 52*P*.
GREGORY, R. A. & TRACY, H. J. (1961). *J. Physiol.* (in the Press).

Two-stage transfer of glycine by the intestine *in vitro*

By H. NEWEY* and D. H. SMYTH. *Department of Physiology, University of Sheffield*

Newey & Smyth (1960*a*) showed that dipeptides present in the intestinal lumen enter the epithelial cells and undergo intracellular hydrolysis. Some of the amino acid formed is absorbed, while some diffuses back into

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the intestinal lumen. Experiments were undertaken to investigate further the cellular mechanisms for peptide and amino-acid transfer.

The preparation was the sac of rat everted intestine (Wilson & Wiseman, 1954). Sacs were incubated in bicarbonate saline containing peptide or amino acid for 30 min in various conditions. With glycine or glycyl-glycine initially present in equimolar concentrations (15·1 mM) in the mucosal fluid, the amounts of glycine transferred to the serosal side were about equal, and with glycyl-glycine about half the glycine formed diffused back into the mucosal fluid. As Newey & Smyth (1960*b*) have suggested a special mechanism for glycyl-glycine entry, a possible explanation was that a transfer mechanism at the luminal border of the cells could transfer both glycine and glycyl-glycine, but in the case of glycyl-glycine one molecule of glycine was split off intracellularly during transfer and returned to the intestinal lumen. This was tested by using $NH_2CH_2CONH^{14}CH_2COOH$ in the mucosal fluid. This showed clearly that the two constituent glycine molecules were equally involved in transfer and back diffusion. This explanation was therefore excluded.

An alternative explanation is that after intracellular hydrolysis about half the glycine formed enters a transfer mechanism in the cell, while half diffuses back into the mucosal fluid. This requires a certain intracellular location of the peptidases and transfer mechanism, and the transfer mechanism must be an 'exit' mechanism on the serosal side of the site of peptidase activity. Further evidence for such a mechanism was shown by anaerobic experiments, in which the proportion of glycine diffusing backwards was greatly increased, while that transferred to the serosal side was diminished by 60%. Dinitrophenol (2×10^{-4} M) had the same effect.

The mechanism was further studied by investigating simultaneous transfer of methionine with glycine or glycyl-glycine. When glycyl-glycine and methionine were initially present, although the amount of glycine formed was reduced (owing to the inhibition of the peptidase by methionine) and methionine transport was not appreciably affected, the fraction of glycine transferred to the serosal side was reduced only by 10%. If, however, glycine and methionine were initially present in the mucosal fluid in equimolar quantities (15·1 mM), glycine transfer was inhibited by 43%. This suggests that the main site of methionine inhibition is not the 'exit' mechanism affected by anoxia.

There may therefore be two stages in glycine transfer; (1) an entry mechanism susceptible to methionine inhibition, and (2) an exit mechanism damaged by anoxia.

REFERENCES

NEWEY, H. & SMYTH, D. H. (1960*a*). *J. Physiol.* **152**, 367–380.
NEWEY, H. & SMYTH, D. H. (1960*b*). *J. Physiol.* **152**, 70–71*P*.
WILSON, T. H. & WISEMAN, G. (1954). *J. Physiol.* **123**, 116–125.

The distances between rods in the cat's retina

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The distance between the centres of adjacent rods in the cat's eye has been measured in a horizontal section of the retina. A cat, 2·5 kg in weight, was killed by coal gas. The eyes were immediately removed and their antero-posterior and equatorial diameters quickly measured before fixation in Bouin's fluid.

Both eyes were dehydrated in cellosolve and embedded in low-viscosity nitrocellulose. Sections were cut horizontally through the posterior part of the retina of one eye. From those sections which passed through the outer segments of the rods enlarged photo-micrographs were prepared; centre to centre distances were then measured on these photomicrographs. The average of several measurements was about 1·3 μ.

An estimate of the shrinkage that had occurred during the preparation of the section was made by measuring the antero-posterior and equatorial diameters of complete vertical sections of the other eye and comparing these values with those measured in the eyes before fixation. The apparent shrinkage was 10%.

The true centre to centre distance of the rods was thus about 1·4 μ. This corresponds to about 50 million rods/cm^2. A previous estimate (Marriott, Morris & Pirenne, 1959) making no allowance for shrinkage gave 46 million/cm^2; if the same correction is applied, the adjusted value is about 38 million/cm^2. The difference between the adjusted estimates is within the limits of experimental error which might be expected when using this method.

According to Østerberg (1935) the maximum density of rods in the human retina is about 16 million/cm^2, and the mean density about 13 million/cm^2.

REFERENCES

Marriott, F. H. C., Morris, V. B. & Pirenne, M. H. (1959). *J. Physiol.* **146**, 179.
Østerberg, G. (1955). *Acta ophthal., Kbh.*, Suppl. 6.

A gastric phase of pancreatic secretion in cats

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Previous workers have not been able to demonstrate the release from the stomach of a humoral agent capable of stimulating pancreatic secretion in the dog (Magee, 1959; Hudock & Lawrence, 1959) or the cat (Harper &

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Vass, 1941). On the other hand it has been shown that extracts of the antral mucosa of man, cat, dog, and pig, when injected intravenously into cats, produce a marked increase in the output of pancreatic enzymes with relatively little effect on the rate of flow of juice (Blair, Harper, Lake, Reed & Scratcherd, 1961). The possibility that there is a gastric humoral phase of pancreatic secretion has therefore been re-investigated.

The experiments were performed on cats anaesthetized with chloralose. The vagus and splanchnic nerves were cut, and in some instances the coeliac ganglia were also removed. The pylorus was occluded and pancreatic juice collected from a cannula in the pancreatic duct. The ligature around the pancreatic duct included the bile duct, to prevent the entry of bile into the duodenum. A steady background flow of pancreatic juice was maintained throughout by repeated intravenous injections of secretin. The stomach was intubated via the oesophagus, and in some experiments separate intubation of the pyloric antrum and of the body of the stomach was achieved by tying a ligature at the junction of the body and the antrum. In some of the experiments 0·01 mg carbaminoyl-β-methyl choline chloride was injected subcutaneously every 15 min as a sensitizing procedure (Grossman, Robertson & Ivy, 1958), but this was not essential for the production of the results to be described.

Various forms of gastric stimulation increased the output of pancreatic amylase with little or no effect on the rate of flow of pancreatic secretion. The stimuli included the introduction of meat digests or extracts into the whole stomach or pyloric antrum, or distension of the stomach or antrum with a balloon, 0·3 osmolar glycine, or 0·005 normal HCl. The most consistently successful experiments were those in which distension was produced with glycine or HCl. The responses to meat extracts could be markedly enhanced if acetylcholine were added to the extracts to a concentration of 0·5%. No increase in the enzyme output was observed when the stimuli were confined to the body of the stomach.

This evidence, combined with that of the results of the injection of antral extracts, supports the view that there is a gastric phase of pancreatic secretion.

We wish to thank Messrs Oxo Ltd. for the supply of meat extracts.

REFERENCES

Blair, E. L., Harper, A. A., Lake, H. J., Reed, J. D. & Scratcherd, T. (1961). *J. Physiol.* **156**, 11*P*.

Grossman, M. I., Robertson, C. R. & Ivy, A. C. (1958). *Amer. J. Physiol.* **153**, 1.

Harper, A. A. & Vass, C. C. N. (1941). *J. Physiol.* **99**, 415.

Hudock, J. & Lawrence, J. W. (1959). *Surgery*, **46**, 682.

Magee, D. F. (1959). *J. Physiol.* **149**, 76*P*.

The effect of neuronal rest on the liberation and uptake of the sympathetic transmitter by the spleen of the cat

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The variability of the proximal convoluted tubules in the kidneys of rats

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Young male white rats (150–200 g), after fasting overnight with free access to water, were treated thus: in nine rats water diuresis (1·0–3·5 ml./15 min) was induced, in a further eight rats water diuresis was induced and then inhibited by subcutaneous Pitressin (vasopressin, Parke, Davis; 25–30 m-u./100 g body wt.), five rats were given the hormone but no water, and five were given neither water nor hormone. Kidneys of all rats were fixed and stained by Bharadwaj & Love's (1959) method for mitochondria, and those of some by LaCour, Chayen & Gahan's (1958) method for lipids, or with Heidenhain's Azan after fixation in Zenker's fluid.

Proximal tubules (*1st segment*). Cells contained a variable number of lipid droplets. Where the latter were close together it was difficult to find mitochondria between them, and it was possible, but not ascertainable, that droplets had formed by the confluence of mitochondrial material. The lumen tended to be smaller in diameter in tubules with numerous conspicuous droplets. Lipid was never certainly seen in the lumen of any tubule.

Proximal tubules (*2nd segment*). In this region the mitochondria, unlike the rods of the first segment, were small spheres, about 0·3 μ in diameter, giving the cytoplasm of many cells a granular appearance. In some tubules the cells were swollen and the mitochondria more widely separated. When the cells were compact in structure the lumen of the tubule varied in cross-section from a full circle down to a stellate crack. If, however, the cells were swollen, the lumen was never widely patent and was often completely occluded. The proportion of tubules in either of these states varied between kidneys.

The frequency of the variations in either part of the proximal convoluted tubule could not be correlated with the procedures to which the rats had been subjected.

REFERENCES

Bharadwaj, T. P. & Love, R. (1959). *Stain. Tech.* **34**, 331–334.
LaCour, L. F., Chayen, J. & Gahan, P. S. (1958). *Exp. Cell. Res.* **14**, 469–485.

Contracture of the denervated rat diaphragm by adrenaline

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It is long known that the twitch of most skeletal muscles produced by nerve or by direct stimulation is enhanced by simultaneous stimulation of its sympathetic nerve or by injection of adrenaline (Orbeli, 1923; Michol, 1930). In extensive studies since then there has been no evidence that adrenaline or sympathetic nerve stimulation by themselves contract innervated skeletal muscle (Bülbring & Burn, 1940; Brown, Bülbring & Burns, 1948; Goffart & Ritchie, 1952). It has been reported, however, that adrenaline produces contracture of denervated facial muscles in the dog (von Euler & Gaddum, 1931; Luco, 1959).

The present experiments demonstrate that adrenaline (4×10^{-6}–4×10^{-7} g/ml.) regularly produces a contracture of the isolated rat diaphragm in Tyrode solution (NaCl, 0·8%; KCl, 0·02%; $CaCl_2$, 0·02%; NaH_2PO_4, 0·005%; $NaHCO_3$, 0·1%; glucose, 0·1%) after degenerative section (10–25 days) of the phrenic nerve. Adrenaline was used as bitartrate and concentrations are expressed as base.

The properties of the adrenaline-induced contracture were: (1) It was slower than that evoked by ACh, was maintained much longer and was never as great as could be obtained with ACh (10^{-6}–10^{-7} g/ml.). (2) It occurred when the diaphragm was tested at 34–37° C but failed to occur at lower temperatures (15–18° C); ACh-induced contractures on the other hand were much greater at the lower temperature. (3) It was unaffected by eserine (5×10^{-6} g/ml.), which increased the effect of ACh; or by D-tubocurarine (10^{-6} g/ml.) which reversibly antagonized the action of ACh. (4) Like the ACh-induced contracture it was greatly reduced or abolished when calcium was omitted from the Tyrode solution; this effect of calcium was reversible.

The denervated rat diaphragm responded by contracture to other choline esters such as butyryl- and dimethylacrylylcholine; also to tetramethylammonium and to nicotine. It did not respond at all to smooth-muscle stimulants such as histamine, 5-hydroxytryptamine, bradykinin, or pilocarpine.

These results suggest that the specificity of the receptors to acetylcholine does not change in skeletal muscle after degenerative section of its motor nerve, and that the receptors for ACh are different from those for adrenaline.

REFERENCES

Brown, G. L., Bülbring, E. & Burns, B. D. (1948). *J. Physiol.* **107**, 115.
Bülbring, E. & Burn, J. H. (1940). *J. Pharmacol.* **68**, 150.
Goffart, M. & Ritchie, J. M. (1952). *J. Physiol.* **116**, 357.

Luco, J. V. (1959). In: *Curare and Curare-like Agents*. Ed. Bovet, D., p. 405. Amsterdam: Elsevier.
Michol, E. (1930). *Z. Biol.* **90**, 313.
Orbeli, L. A. (1923). *Bull. Inst. Sci. Lesshaft, Petrograd*, **6**, 194.
von Euler, U. S. & Gaddum, J. H. (1931). *J. Physiol.* **73**, 54.

The assay, preparation and properties of urogastrone

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Inhibition of acid gastric secretion by extracts of human urine was first reported in 1939 and the active principle was called 'urogastrone' by Gray, Culmer, Wieczorowski & Adkison (1940). The purification of urogastrone has been studied most recently by Gregory (1955), but progress in its characterization has been impeded by lack of a quantitative bioassay method.

We have developed a method of assay based on the perfused rat-stomach preparation (Ghosh & Schild, 1958). The stomach of a urethanized rat is perfused with a dilute phosphate–citrate buffer, the pH of which is continuously recorded. Intravenous injections of 1 μg carbachol cause a fall of pH of the perfusate lasting 45–60 min. This acid response can be antagonized by the intravenous administration of urogastrone 5 min earlier and the log. dose–effect relationship for urogastrone is linear over a four-fold dose range. A self-contained assay block of four doses of inhibitor may be obtained with each animal. This involves ten to twelve injections of carbachol including recovery doses of carbachol alone between injections of the inhibitor, the whole experiment lasting about 10 hr.

Urogastrone is extracted from male urine by a modification of the procedure described by Johnsen (1955) for the bulk extraction of urinary gonadotrophin. The active material is adsorbed on a zeolite column and differentially eluted with a buffered ammonium acetate–alcohol solution yielding 3–8 mg/l. urine of a tan brown Biuret-positive, Molisch-positive powder which inhibits acid secretion in a rat in doses of 0·5–2 mg.

The urogastrone activity is retained on boiling at pH 6·8 for 30 min, but is rapidly lost on boiling in N-HCl, with a half time of approximately 15 min. The activity is gradually destroyed by incubation at 38° C with chymotrypsin at pH 7·8 and with pepsin in N/10 HCl. There is an exponential decline of activity to about 5–10% in 24 hr on incubating 20 mg crude urogastrone with 1 mg of a purified preparation of either of these enzymes. Similar incubation with trypsin at pH 7·8 causes only slight loss of activity which may be due to contamination with chymotrypsin. Treatment of crude urogastrone with reducing agents such as cysteine (125 mM) and

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sodium sulphite (50 mM) produces considerable reduction of activity. It is concluded that urogastrone is probably a polypeptide and that disulphide bonds are important for its activity.

REFERENCES

GHOSH, M. N. & SCHILD, H. O. (1958). *Brit. J. Pharmacol.* **13**, 54–61.

GRAY, J. S., CULMER, C. U., WIECZOROWSKI, E. & ADKISON, J. L. (1940). *Proc. Soc. exp. Biol., N.Y.*, **43**, 225–228.

GREGORY, R. A. (1955). *J. Physiol.* **129**, 528–546.

JOHNSEN, S. G. (1955). *Acta endocr. Copenhagen*, **20**, 101–105.

Excitability changes following subliminal stimulation of frog skin mechanoreceptors

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Subliminal mechanical or electrical conditioning stimuli, applied by means of a metal stylus to the outer surface of a frog skin-nerve preparation, gave rise to changes in excitability of single receptors, revealed by brief mechanical or electrical test stimuli. The other electrode consisted of a silver plate (1 cm diameter) in contact with the inner surface. The effects observed depended on the intensity and duration of the subliminal conditioning stimulus, and in the case of an electrical stimulus on its polarity. Brief ($\gtrsim$ 2 msec) mechanical or cathodal stimuli produced a very short phase of supernormality followed by a period of subnormality not exceeding about 10 msec in most cases, but occasionally lasting for 50 msec or more. With longer conditioning stimuli (20–30 msec) pronounced excitability changes were found only at the beginning and end of the pulse. For a long subliminal mechanical pulse there was a brief sharp rise of excitability at the beginning and a smaller rise at the end of the pulse; during the pulse there was a low plateau of increased excitability. For a long subliminal cathodal pulse there was a sharp rise at the onset and a sharp fall at the end; during the pulse the excitability was above normal but gradually declining. For a long anodal pulse the effects were the opposite of these. With stronger electrical stimuli a spike was fired at the onset of a cathodal pulse or at the end of an anodal pulse. With long mechanical pulses the off-threshold was about five times the on-threshold.

The results support the view that mechanical stimulation causes depolarization of the receptors, which show dynamic and static phases of excitability change reminiscent of the dynamic and static phases of the radial displacement of lamellae of the Pacinian corpuscle (Hubbard, 1958) and of the generator potential of the frog's muscle spindle (Katz, 1950). The off-response is foreshadowed by a sharp rise in excitability at the end

of a long subliminal mechanical stimulus. The brief dynamic excitability changes at the beginning and end of a long mechanical pulse may be a reflexion of the property of rapid adaptation characteristic of many frog skin mechanoreceptors.

REFERENCES

HUBBARD, S. J. (1958). *J. Physiol.* **141**, 198–218.
KATZ, B. (1950). *J. Physiol.* **111**, 261–282.

Histamine formation in the rat

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Kahlson (1960) has postulated that the histamine-forming capacity of rat tissues is related to growth. The following results, obtained using a modification of Waton's method (1956), cast doubt on this theory.

(1) The histidine decarboxylase activity of the foetal liver was less than that of the adult liver when calculated per tissue instead of per gram, except during the last 2 days of pregnancy. Little or no activity was detected in the foetus before the 13th day of gestation or in the young rat before the 10th day of life.

(2) Different optimal conditions were required for the determination of histidine decarboxylase activity in the foetal liver, the adult liver and the adult pyloric stomach of normal rats (Telford & West, 1961*a*, *b*). On treatment with histamine liberators or glucocorticoids, and on exposure to cold, the enzyme activity of the adult liver was reduced but that of the pyloric stomach was increased.

(3) The high histidine decarboxylase activity of a transplantable rat hepatoma (Mackay, Marshall & Riley, 1960) was confirmed, but even greater activity was found when portions were necrotic. The tumour enzyme required for optimal activity conditions resembling those for the foetal enzyme.

(4) The histidine decarboxylase activity of the liver was reduced during the period of maximal regeneration after partial hepatectomy. Urinary histamine was decreased.

These results suggest there are 3 types of histidine decarboxylase in the rat, none of which may be related to growth. Thus, that in the foetus may produce histamine to increase the foetal and placental blood supply, and to inhibit the mother's uterine movement; that in the adult liver, which shows very high *in vitro* activity, may produce histamine to function throughout the body; and that in the adult pyloric stomach may produce histamine to function as a local stimulus of gastric secretion. The hepatoma

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enzyme may produce histamine to increase the blood supply to the tumour.

When this work was extended to the rabbit and guinea-pig the foetal histidine decarboxylase activity was found to be low, suggesting that in these species also the enzyme may not be related to growth.

REFERENCES

KAHLSON, G. (1960). *Lancet*, **278**, 67–71.
MACKAY, D., MARSHALL, P. B. & RILEY, J. F. (1960). *J. Physiol.* **153**, 31*P*.
TELFORD, J. M. & WEST, G. B. (1961*a*). *J. Pharm. Lond.*, **13**, 75–82.
TELFORD, J. M. & WEST, G. B. (1961*b*). *J. Physiol.* **155**, 16*P*.
WATON, N. G. (1956). *Brit. J. Pharmacol.* **11**, 119–127.

Folic acid requirements in man

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Folic acid requirements in man are not yet known (Girdwood, 1959). The most satisfactory method of determining the folic acid stores of an individual is probably by following the excretion of formimino glutamic acid (FIGlu) in the urine after an oral dose of histidine (Tabor, Silverman, Mehler, Daft & Bauer, 1953). This compound can be separated in the urine by high-voltage electrophoresis (Knowles, Prankerd & Westall, 1960).

In normal individuals after a 20 g dose of histidine only traces of FIGlu may be excreted between 5 and 10 hr. By collecting urine for 6 hr following the histidine the FIGlu concentration becomes too low to be detectable in normal persons, but is sufficiently high in folic-acid-deficient individuals. Estimation of FIGlu clearance shows that, unlike other amino acids, the clearance of FIGlu is high, about 120 ml./min.

A study has been made of four patients fed on an artificial liquid diet which appears to provide a complete replacement of all known dietary requirements except folic acid. Estimation has shown that its folic acid content is less than 0·5μg/kg (Fleming, A., personal communication). On such a diet individuals showed an abnormal excretion of FIGlu after 4–5 weeks. A further patient fed on the same diet with the addition of foods containing 2–5 μg of folic acid daily did not excrete excess FIGlu after histidine (30 g) loading even after a period of 3 months. Thus it would appear that the minimum folic acid requirements in man are of the order of 5 μg per day, which would suggest that the total body stores do not normally exceed 0·2 mg. After treatment with folic acid the FIGlu excretion returns to normal, but in contrast to the rapid effect of folic acid on a megaloblastic bone marrow, a matter of hours, there is a delay of several days before FIGlu excretion returns to normal.

REFERENCES

GIRDWOOD, R. H. (1959). *Brit. med. Bull.* **15**, 14.

TABOR, H., SILVERMAN, M., MEHLER, A. H., DAFT, F. S. & BAUER, H. (1953). *J. Amer. chem. Soc.* **75**, 756.

KNOWLES, J. P., PRANKERD, T. A. J. & WESTALL, R. G. (1960). *Lancet*, **279**, 347.

Perfusion of the giant nerve fibres of *Loligo*

By P. F. BAKER, A. L. HODGKIN and T. I. SHAW. *The Laboratory of the Marine Biological Association, Plymouth*

As has been mentioned in a preliminary report (Baker & Shaw, 1961), giant nerve fibres give propagated action potentials after the axoplasm has been replaced with an artificial solution. The method consisted in cannulating an axon at one end, squeezing out axoplasm through the other end and re-inflating the flattened sheath with isotonic solutions such as potassium sulphate, methylsulphate or isothionate. After introducing an internal electrode, the quantitative effects of different solutions were determined by applying fluid through a cannula at one end of the fibre and withdrawing it from the other, often through a second cannula.

In spite of the drastic treatment, axons filled with isotonic potassium solutions (plus phosphate buffer, pH 7–8) remained excitable for several hours and were capable of carrying a large number of impulses, e.g. 3×10^5 at 16° C. The action potential (90–110 mV) and the resting potential (50–70 mV) were of the usual magnitude and the shape of the spike was similar to that in an intact fibre.

Changes in the composition of the internal solution gave rapid and reversible effects of the kind expected from the ionic theory. Partial replacement of K by Na first reduced and then abolished the action potential with little change in resting potential; complete replacement of K by Na reduced the resting potential by 40–50 mV. With isotonic KCl outside and isotonic NaCl inside, the normal resting potential was reversed, the internal solution being 50–60 mV positive to the external solution. The resting potential was close to zero if similar solutions were placed on either side of the membrane.

The communication will be illustrated by histological preparations which show that extrusion and subsequent perfusion remove nearly all the axoplasm.

REFERENCE

BAKER, P. F. & SHAW, T. I. (1961). *J. Mar. Biol. Ass. U.K.* **41** (in the Press). Report for 1960–61.

Effects of afferent stimulation on reticulo-spinal neurones

By J. H. Wolstencroft. *Department of Physiology, University of Leeds*

Extracellular action potential recordings have been made from single neurones in the medial reticular formation of the medulla of decerebrate cats with glass micro-electrodes of tip diameter 1–10 μ. Reticulo-spinal neurones have been identified by antidromic stimulation in the spinal cord. Many of these neurones respond to impulses generated by both nociceptive cutaneous and muscle afferent stimulation as shown by physiological methods and by electrical stimulation of muscle and cutaneous nerves supplying the four limbs and the neck. Reticulo-spinal neurones responding to these stimuli can be divided into two groups: (i) those excited by all applied stimuli, (ii) those showing patterns of excitation and inhibition. For both groups the receptive field covers a very wide area of the body.

The effect of infusions of noradrenaline on the O_2 consumption of young animals

By J. W. Scopes and J. P. M. Tizard. *The Nuffield Institute for Medical Research, University of Oxford*

Von Euler & Söderberg (1958) showed that intravenous infusion of adrenaline or noradrenaline into anaesthetized adult cats at a rate of 1–2 μg/min induced shivering and caused an increase in body temperature. Moore & Underwood (1960) found that subcutaneous injection of very large doses of noradrenaline into infant kittens caused an increase in O_2 consumption and rectal temperature. The object of the present experiments was to determine the threshold dose of noradrenaline required to cause an increase in O_2 consumption in young animals, when given by intravenous infusion.

In rabbits and kittens, from birth up to 1 month of age, O_2 consumption was measured either under general anaesthesia (chloralose or pentobarbitone) or after introduction of a venous catheter under local anaesthesia. Under general anaesthesia, tidal air, respiratory rate and blood pressure were also recorded. An infusion of noradrenaline at a rate of 1 μg/kg.min caused a rise in O_2 consumption in animals not under general anaesthesia. In anaesthetized kittens and rabbits rather larger doses were required; 2 μg/kg.min was almost always effective. These doses caused a large rise in blood pressure, an increase in the minute volume of respiration and an increase in rectal temperature. Occasionally, in a cold environment and in older kittens visible shivering was induced.

At environmental temperatures below the neutral range, the O_2 consumption still rose on infusion of noradrenaline, but the magnitude of

the rise seemed to be dependent on the degree of stability of body temperature. Hypoxia (breathing 8–10 % O_2) greatly diminished the response, but hypercapnia (breathing 5 % CO_2 in air) did not diminish the rise in body temperature consequent on the infusion.

REFERENCES

VON EULER, C. & SÖDERBERG, U. (1958). *Acta physiol. scand.* **42**, 112–129.
MOORE, R. E. & UNDERWOOD MARY C. (1960). *J. Physiol.* **150**, 13–14*P*.

Cardiac output and forearm blood flow during intravenous infusion of adrenaline in man

By M. J. ALLWOOD, E. W. KECK, R. J. MARSHALL and J. T. SHEPHERD.
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The recovery of electrical activity in the optic tectum of the frog during early regeneration of the optic nerve

By M. JACOBSON. *Department of Physiology, University of Edinburgh*

The left optic nerve was cut close to the optic chiasma in 40 adult frogs (*Rana temporaria*). After 20–120 days these frogs were used for mapping the projection of the left visual field on to the right optic tectum. Action potentials evoked in response to a spot of light subtending an angle of 10′ in the frog's visual field were recorded by means of a steel microelectrode on the tectum and amplified in the usual manner. The responses consisted of groups of action potentials from many units.

No evoked responses were obtained from nine frogs. In sixteen frogs the normal retinotopic projection on to the optic tectum had been restored after optic nerve regeneration. In fifteen frogs responses were not obtained from their normal positions, but could be evoked only from one or two localized regions of the visual field. In eight frogs there was a small region in the nasal half-field from which a light evoked responses in the lateral half of the tectum, and another region in the temporal half-field from which responses were evoked in the medial half of the tectum (Fig. 1). In five frogs only the nasal responsive region was present, and in two frogs only the temporal responsive region. In these cases the responsive region projected on to the appropriate half of the optic tectum, and no evoked potentials could be recorded from the other half of the tectum.

These results indicate that optic axons regenerate first from a localized region in each half-retina rather than simultaneously from all parts of the retina. Though each half-retina had established functional connexions with the appropriate half of the optic tectum, a more detailed retinotopic projection was not apparent. It is suggested that segregation of optic axons in the optic tract may have occurred, so that axons from the nasal

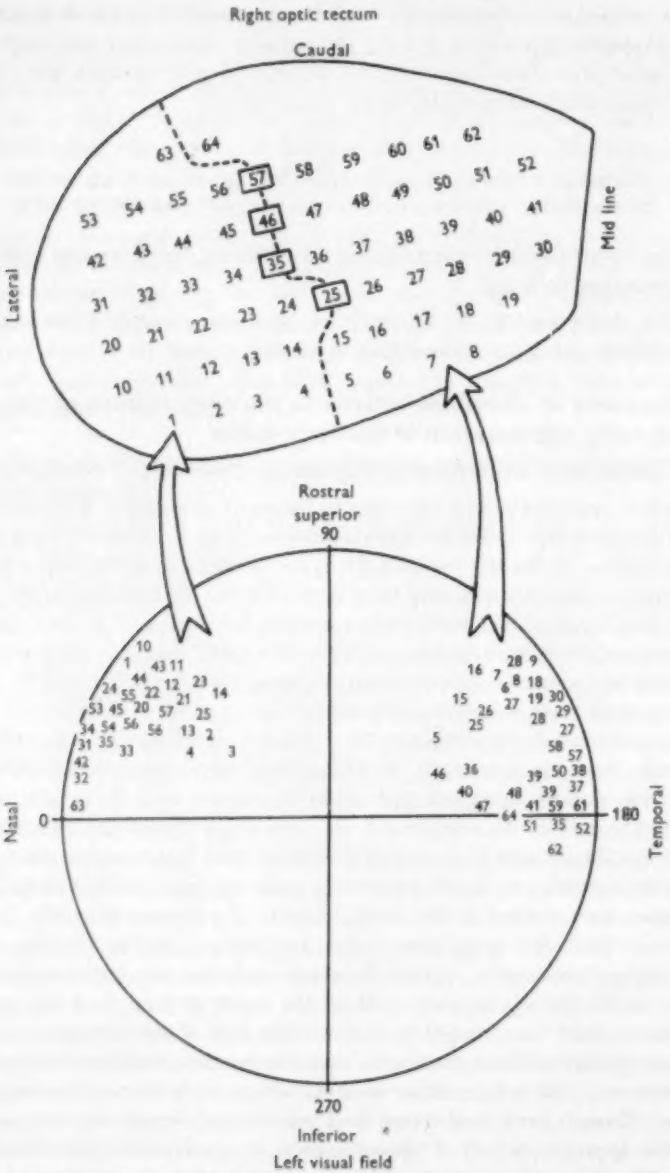

Fig. 1. The projection of the left visual field on to the right optic tectum 68 days after transection of the left optic nerve. The outline of the right optic tectum shows electrode position at which action potentials were evoked in response to a light at the positions shown in the visual field.

and temporal half-retinae regenerated selectively into the medial and lateral brachia of the marginal optic tract.

Interaction in the visceral sensory areas of the cerebral cortex

By P. P. NEWMAN. *Department of Physiology, School of Medicine, Leeds*

The discharge patterns of single cortical neurones evoked by stimulation of a splanchnic nerve have been previously reported (Newman, 1961). It was observed that increases in intensity of nerve stimulation reduced the mean latency of the first spike, increased the number of spikes per discharge and increased the probability of response.

In cats under light pentothal anaesthesia forms of occlusive interaction from two peripheral sources have been studied. The animals were paralysed with Flaxedil and artificially ventilated with 100% oxygen. It was found that many cortical units evoked by splanchnic stimulation could also be driven by touching or stroking the abdominal skin. In order to demonstrate possible interaction between the two pathways a pair of needle electrodes was inserted into the skin on the same side as the splanchnic nerve. Temporal separation of the stimuli was effected by means of two pulse generators of variable delay which were triggered to the sweep of an oscilloscope. When a unit which responded to splanchnic stimulation was isolated, previous stimulation of the skin resulted in a striking alteration of the cortical response.

In one type of experiment a conditioning stimulus was delivered to the skin and a testing stimulus to the splanchnic nerve. When the interval between the two stimuli was more than 60 msec there was no observable interaction. As the conditioning–test interval was gradually reduced the unit responded less frequently to splanchnic stimulation and finally ceased to fire unless the conditioning stimulus was switched off. At the same time the mean latency of the test response progressively increased until complete occlusion occurred. In a second type of experiment reversing the sequence of stimulation yielded comparable results. The unit failed to respond to stimulation of the skin when the conditioning–test interval was less than 30 msec.

It may be concluded from these experiments that splanchnic and cutaneous pathways converge in the central nervous system and influence the same cortical neurone. The most prominent change observed was occlusion of the test response. The results give support to the earlier investigations of Amassian (1952) on interaction in the somato-visceral projection system.

REFERENCES

AMASSIAN, V. E. (1952). *Res. Publ. Ass. nerv. ment. Dis.* **30**, 371–402.
NEWMAN, P. P. (1961). *J. Physiol.* (in the Press).

The infusion of brain extracts into the anterior pituitary gland and the secretion of gonadotrophic hormone

By H. J. Campbell, G. Feuer, J. Garcia and G. W. Harris. *Departments of Neuroendocrinology and Biochemistry, Institute of Psychiatry, The Maudsley Hospital, London, S.E. 5*

Much evidence exists that the hypothalamus regulates anterior pituitary activity by means of a humoral mechanism involving the hypophysial portal vessels of the pituitary stalk. These vessels originate in the median eminence of the tuber cinereum. Extracts obtained from median eminence tissue, and from other parts of the brain (cerebral cortex, caudate nucleus and corpus callosum) have been slowly infused (0·134 ml. in 2 hr) into the

Table 1. The effect of intrapituitary and intravenous infusions of various substances on ovulation in the rabbit

		Route of administration			
		Intrapituitary		Intravenous	
Substance	Dose*	No. of expts.	Rabbits ovulating	No. of expts.	Rabbits ovulating
Extracts of median eminence	100	—	—	7	1
	50	—	—	14	4
	20	25	16	10	0
	10	14	10	—	—
	5	13	9	—	—
	2·5	23	12	—	—
	1·0	7	0	—	—
Extract of cerebral cortex, caudate nucleus and corpus callosum	50	—	—	2	0
	20	24	1	—	—
Vasopressin, oxytocin, substance P, adrenaline, histamine, serotonin	—	49	2	—	—

* Dose expressed as mg wet weight of tissue.

anterior pituitary gland of isolated female rabbits by means of platinum cannulae (S.W.G. 25) implanted one week previously. During infusion the animals were conscious and unrestrained. The brain tissue, obtained from rabbits and steers, was extracted with 0·5% acetic acid at 100° C for 5 min. After centrifuging, the supernatant was neutralized, made isotonic and kept frozen until used. Infusions have also been made of substances known to be present in the hypothalamus (synthetic lysine vasopressin, synthetic oxytocin, substance P and neutralized solutions of adrenaline, histamine and serotonin). The brain extracts have also been slowly infused (2·1 ml. in 2 hr) *intravenously*.

It may be seen from Table 1 that (*a*) intrapituitary infusion of control solutions was followed by ovulation in only 3 out of 73 experiments, (*b*) intrapituitary infusion of median eminence extract (2·5–20·0 mg wet weight of tissue) was followed by ovulation in 47 out of 75 experiments, and (*c*) intravenous infusion of median eminence extract (20 mg) did not excite ovulation, although infusions at a higher dose (50–100 mg) resulted in ovulation in 5 out of 21 experiments. It seems unlikely that the effect of intrapituitary infusion of median eminence extract may be explained in terms of pituitary damage or contamination of the extract with gonadotrophic hormone. Median eminence tissue probably contains a specific substance which stimulates the release of gonadotrophic hormone from the anterior pituitary gland.

Attempts to isolate pyruvylcholine

By E. W. Gill, J. A. Parsons and W. D. M. Paton. *Department of Pharmacology, University of Oxford*

We have attempted to prepare pure pyruvylcholine in order to resolve doubts about the identity of a naturally occurring choline ester. The latter was isolated from the abdomen of the Garden Tiger Moth (*Arctia caja*, Linn.) and seems quite distinct from the choline ester reported in the thorax of this insect by Bisset, Frazer, Rothschild & Schachter (1960). It may be acetylcholine itself, and indeed by repeated chromatography we have isolated an ester as a crystalline hexanitrodiphenylamine salt which is identical in m.p. and mixed m.p. with the corresponding derivative of acetylcholine.

None the less, slight differences in activity on repeated assay and in R_F on chromatography of the original extracts made us wish to exclude the possibility that some other ester might be converted into acetylcholine during extraction.

Pyruvylcholine has a special place among choline esters in that it was the only one which Chang & Gaddum (1933) in their classical paper were not able to distinguish clearly from acetylcholine by parallel assay. They obtained the compound from H. W. Dudley.

We have repeated in detail the published preparations by Le Heux (1921) and Dudley (1931), purifying the products by repeated chromatography and isolating them as the hexanitrodiphenylamine and tetraphenylboron salts.

We have also repeated the published preparation of pyruvylcholine by Horenstein & Pählicke (1938), but were unable to find any evidence for the existence of pyruvylcholine in the product.

From the preparations of Le Heux and Dudley, which are substantially identical, we isolated an ester fraction which in spite of thrice-repeated chromatography was still impure, as judged by unsharp melting points of the two derivatives. On repeated recrystallization the melting points of these compounds rose and sharpened. They finally became identical with those of corresponding derivatives of pure acetylcholine and were undepressed when mixed with authentic samples of the latter compounds. On bio-assay (guinea-pig ileum) small samples also gave specific activities in excellent agreement with corresponding derivatives of acetylcholine.

The persistently depressed m.p. of the chromatographically homogeneous samples is strong evidence for the presence of at least two compounds with closely similar R_F. We have been repeatedly impressed by the unreliability of paper chromatography without supporting evidence as a method for *identification* of closely related choline esters. Although increase in chain length tends to raise the R_F, the presence of polar groups tends to lower it.

It appears that pyruvylcholine has never been obtained pure and free from acetylcholine, and should be regarded as a substance of unknown biological activity. Parallel assay as a criterion of identity among choline esters is now left without known exception.

REFERENCES

Bisset, G. W., Frazer, J. F. D., Rothschild, M. & Schachter, M. (1960). *Proc. Roy. Soc.* B, **152**, 255–262.
Chang, H. C. & Gaddum, J. H. (1933). *J. Physiol.* **79**, 255–285.
Dudley, H. W. (1931). *J. Chem. Soc.* **1**, 763–769.
Horenstein, H. & Pählicke, H. (1938). *Ber. dtsch. chem. Ges.* **71**, 1644–1657.
Le Heux, J. W. (1921). *Pflüg. Arch. ges. Physiol.* **190**, 280–300.

Seasonal changes in the insulin stimulated efflux of galactose from the isolated perfused rat heart

By R. B. Fisher* and D. A. B. Young.† *Department of Biochemistry, University of Oxford*

Fisher & Lindsay (1956) showed that galactose transport into the rat heart is insulin-sensitive. The rate of transport is too great to obtain an accurate time course. It is, however, possible to follow the efflux from a heart previously loaded with the sugar, by collecting coronary effluent in short timed periods. With this technique an insulin effect is readily observed.

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The time course of the efflux differs from that of efflux from the cells owing to lag in turnover of sugar in the extracellular fluid and possibly owing to capillary wall hindrance. Experiments with inulin, raffinose and sucrose show that both of these effects occur. Semilogarithmic plots of

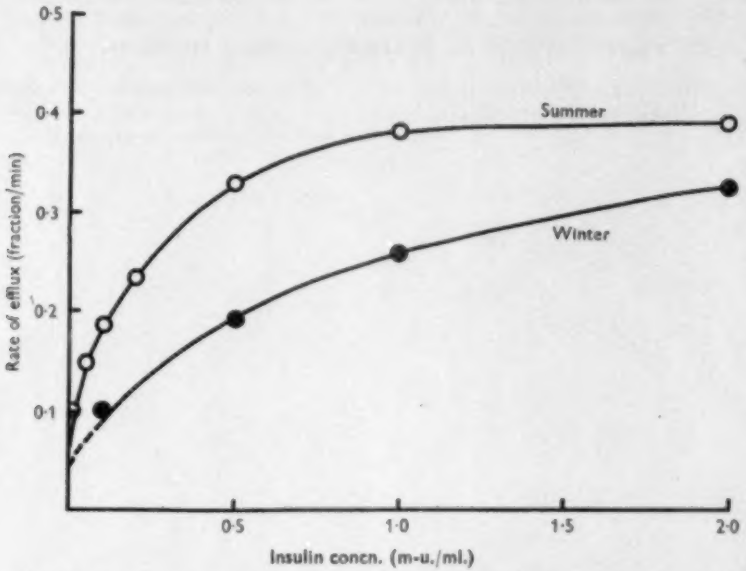

Fig. 1. The dose–response curves for insulin in summer and winter. The response of the tissue is measured as the rate of efflux of galactose, that is the fraction of the heart content emerging in the fourth minute.

efflux show a faster and a slower component, the latter being dominant by the fourth minute. We have used as a measure of efflux rate the fractional fall in heart galactose content during the fourth minute after transfer from perfusion with galactose-containing (1 g/100 ml.) to galactose-free Krebs bicarbonate medium.

Dose–response curves with insulin showed a marked seasonal variation (Fig. 1). High carbohydrate feeding had no effect on the winter response.

For insulin concentrations of 0·2 m-u./ml. upwards, the dose–response curves were Michaelis–Menten in form. (At lower insulin concentrations there is doubt about the correction to be made for depletion of sugar in the extracellular space: at higher concentrations this correction can be neglected.)

The form of the curves suggests that insulin may act by direct combination with the sugar carrier in the membrane to produce a more efficient carrier. The seasonal effect suggests that some unknown factor can interfere competitively with this combination.

REFERENCE

FISHER, R. B. & LINDSAY, D. B. (1956). *J. Physiol.* **131**, 526–541.

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